

Application of molecular biological techniques for detection of epizootic hemorrhagic disease virus (EHDV-318) recovered from a sentinel calf in central Sudan

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Abstract

Epizootic hemorrhagic disease virus (EHDV), isolate 318 (EHDV-318), an untyped virus recovered from a sentinel calf herd at the Khartoum University farm in central Sudan, was characterized using molecular biological techniques. With dot blot hybridization technique, a cDNA probe derived from genome segment 6 of EHDV-2 (Alberta strain) hybridized with RNA from EHDV-318. Application of serogroup-specific EHDV polymerase chain reaction (PCR) to EHDV-318 RNA resulted in specific amplification of a 387 bp PCR product. Amplification product was visualized on ethidium bromide-stained agarose gel. Specificity of the PCR products was confirmed by chemiluminescent hybridization with a non-radiolabelled internal probe. No amplification product or hybridization signal was detected when the serotype-specific EHDV-1 or EHDV-2 PCR-based assays were applied to RNA from EHDV-318. The scientific data presented in this study indicated that cDNA probes and serogroup-specific PCR-based assay can be used to classify the virus as a member of EHDV serogroup, and as serotypically distinct from EHDV-1 and EHDV-2.

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1. Introduction

Epizootic hemorrhagic disease virus (EHDV) is an insect-transmitted double stranded (ds) RNA Orbivirus in the family Reoviridae (Borden et al., 1971; Fenner et al., 1974). The virus has a worldwide distribution and exists in at least ten distinct serotypes (Gorman, 1992). In central Sudan the incidence rates of EHDV showed a marked seasonal pattern, with the infection level being higher in the rainy months which coincided with a high activity of the vector, *Culicoides spp* (Mohammed and Taylor, 1987). EHDV, isolate 318 (EHDV-318), was first isolated from a sentinel calf herd at the Khartoum University farm, Shambat (Mohammed and Mellor, 1990). Subsequently, the virus was isolated from a calf in the Sultanate of Oman (Al-busaidy and Mellor, 1991). Recently, EHDV-318 was reported to be a contaminant of genetically-engineered Chinese hamster ovary (CHO) cells in Bahrain (Rabenau et al., 1993).

EHDV serotype 1 (EHDV-1) and serotype 2 (EHDV-2) cause an often fatal hemorrhagic disease in North American white-tailed deer (*Odocoileus virginianus*) (Shope et al., 1955 and Shope et al., 1960; Nettles et al., 1992). Very little information is available on the impact of EHDV on domestic or wild ruminants in the Sudan. The sale of livestock and associated germplasm may be affected in the international market, unless the animals are certified free of infection by conventional virus isolation or serology (Osburn et al., 1994). Recently, we described reproducible, simple, rapid and sensitive PCR-assays for detection of EHDV-isolates that were serogroup-specific (Aradaib et al., 1994a; Aradaib et al., 1994b; Aradaib et al., 1995b) and serotype-specific identification in cell culture and a variety of clinical samples (Aradaib et al., 1995c; Aradaib et al., 1995d).

In the present study, the recently reported untyped EHDV-318 (Mohammed and Mellor, 1990) was compared with those of North American serotypes of EHDV-1 and EHDV-2 using dot blot hybridization and PCR technology.

2. Materials and methods

2.1. Cell culture and virus propagation

The EHDV-318 (Faculty of Veterinary Science, University of Khartoum, Sudan), EHDV serotype 1 (EHDV-1) and 2 (EHDV-2) (Arthropod-Borne Animal Disease Research Laboratory, Laramie, WY) were used in this study. The viruses were isolated and processed as described previously (Aradaib, 1995a). All viruses were propagated on confluent monolayers of BHK-21 cells. The infectious material was harvested and centrifuged at $1,500 \times g$ for 30 min and the cell pellet was used for the dsRNA extraction.

2.2. *Extraction of viral nucleic acid from infected cell culture*

The EHDV dsRNA was extracted from the infected cells as previously described (Aradaib et al., 1994a). Total nucleic acid was ethanol-precipitated. Viral dsRNA was purified by differential lithium chloride precipitation, and resuspended in 100 μ l double distilled water, and quantified using a spectrophotometer at 260 nm wavelength.

2.3. *Synthesis of the cDNA probe and dot blot hybridization*

A PCR-generated 224 bp cDNA probe derived from genome segment 6 of EHDV-2 (Alberta strain), representing 15% of the full length genome, was used for chemiluminescent hybridization of the blots (Aradaib et al., 1994a). Briefly, the primers were synthesized on a DNA synthesizer (Milligen/Biosearch, A division of Millipore, Burlington, MA) and purified using oligo-pak oligonucleotide purification columns (Glen Research Corporation, Sterling, VA) as per manufacturer's instructions. The probe was purified using DNA binding beads (Mermaid Kit, Bio 101, La Jolla, CA) according to the manufacturer's instructions and used for hybridization of the blotted nucleic acids. Chemiluminescent hybridization was performed as described by Aradaib et al. (1994a).

2.4. *Polymerase chain reaction*

The PCR protocols used in this study for the detection of EHDV serogroup was previously described by Aradaib et al. (1994a). Specific identification of EHDV-1 by PCR-based detection assay was described previously (Aradaib et al., 1995c). PCR specific detection of EHDV-2 was described in detail in a previous report (Aradaib et al., 1995d).

Twenty microliters from each PCR reaction containing amplified product were loaded onto gels of 1.5% SeaKem agarose (FMC Bioproducts, Rockland, ME) and electrophoresed. The gels were stained with ethidium bromide and visualized under UV light (Aradaib et al., 1994a; Aradaib et al., 1995b; Aradaib et al., 1995c).

2.5. *Southern blot hybridization*

Southern blot hybridization was performed basically as previously described (Aradaib et al., 1994a). Briefly, agarose gels from PCR reaction were denatured, neutralized, and the DNA fragments transferred to nylon membranes (Schleicher and Schvell, Woburn, MA) under vacuum at 50 mm Hg (Vacugene XL Vacuum Blotting System, Pharmacia LKB Biotechnology, Piscataway, NJ). Southern blotted nucleic acids were UV-cross linked to the nylon membrane. Chemiluminescent hybridization was performed using a commercial kit (ECL direct nucleic acid labeling and detection system, Amersham Corporation, Arlington Heights, IL) 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 Na Cl at 42°C for 1 h. The labeled probe was added to the hybridization buffer and the membranes were hybridized overnight. After post hybridiza-

1 2 3 4



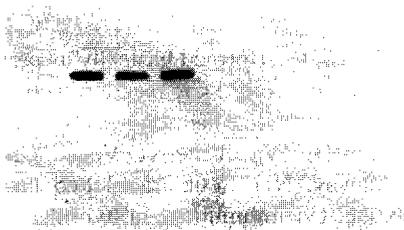
Fig. 1. The sensitivity of the PCR-generated cDNA probe. Lane 1–3: hybridization signals with RNA from EHDV-1, EHDV-2 and EHDV-318, respectively. Lane 4: no hybridization signal was observed with total nucleic acid from BHK-21 cell control.

MW 1 2 3 4



A

MW 1 2 3 4



B

Fig. 2. Detection of EHDV-1, EHDV-2 and EHDV-318 by the serogroup-specific PCR-based assay. (A) Visualization of the 387 bp specific-EHDV PCR product on ethidium bromide-stained agarose gel from 1.0 µg of RNA of EHDV-1, EHDV-2 and EHDV, isolate 318 (untyped). Lane MW: molecular weight marker; lane 1: EHDV-1; lane 2: EHDV-2; lane 3: EHDV-318; lane 4: BHK cell control. (B) Southern blot with chemiluminescent hybridization of the above gel.

tion washing detection reagents were applied to the membranes for 1 min. The membranes were then sealed in saran wrap and exposed to X-ray film for 1–30 min with an intensifying screen.

3. Results

The dot blot hybridization technique, using PCR-generated cDNA derived from genome segment 6 of EHDV-2 (Alberta strain), showed positive hybridization signals with RNA from EHDV-1, EHDV-2 and EHDV-318. No hybridization signal was detected from total nucleic acid of the BHK-21 cell control (Fig. 1).

EHDV-318 RNA was reproducibly and specifically detected by the EHDV serogroup-specific PCR-based assay. The serogroup-specific 387 bp PCR product was visualized on ethidium bromide-stained gel from 1.0 µg RNA of EHDV-1, EHDV-2 and the untyped isolate of EHDV-318 used in this study (Fig. 2A). The identity of the amplified products was confirmed by southern blot with chemiluminescent hybridization (Fig. 2B).

No amplification product or hybridization signals were detected when the serotype-

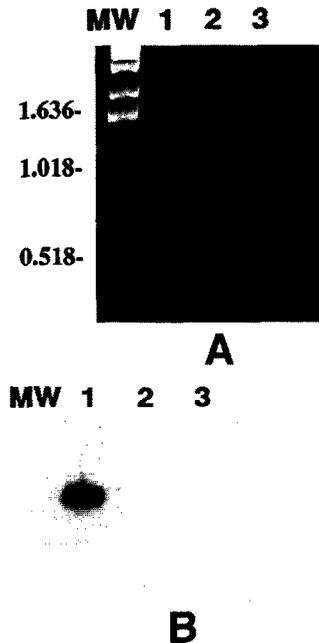


Fig. 3. (A) Visualization of the specific EHDV-1 PCR product (826 bp) from EHDV-1 RNA on ethidium-bromide-stained agarose gel from 1.0 µg of EHDV-1 RNA but not from EHDV-2 or EHDV-318. Lane MW: molecular weight marker; lane 1: EHDV-1 lanes 2: EHDV-2; lane 3: EHDV-318. (B) Southern blot with chemiluminescent hybridization of the above gel.

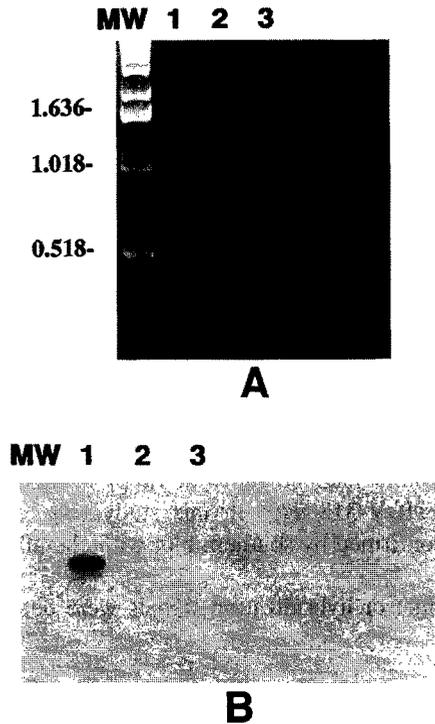


Fig. 4. Specificity of the PCR for EHDV-2 RNA. The specific 1052 bp EHDV-2 PCR product was detected from EHDV-2 RNA only. Amplification products were not detected from EHDV-1 or EHDV-318 RNA. Lane MW: molecular weight marker; lane 1: 1.0 pg EHDV-2; lane 2: 1.0 pg EHDV-1 RNA; lane 3: 1.0 pg EHDV-318 RNA.

specific EHDV-1 (Fig. 3A and B) or EHDV-2 (Fig. 4A and B) PCR-based assays were applied to RNA from EHDV-318.

4. Discussion

Epizootic hemorrhagic disease virus (EHDV), isolate 318 (EHDV-318), described in this study, was recovered from a sentinel calf herd in Shambat, central Sudan (Mohammed and Mellor, 1990). At least 2 serotypes of EHDV designated EHDV-5 and EHDV-318 are known to be enzootic in the Sudan (Mohammed and Taylor, 1987; Mohammed and Mellor, 1990). Very little information is available regarding the epidemiology of Orbiviruses originally isolated in Africa. Further studies on these Orbiviruses are necessary to determine their biology, ecology and molecular epidemiology.

The recently reported EHDV serogroup-specific PCR-based assay using well-characterized serogroup-specific primers derived from segment 6 of EHDV-2, which codes for NS1, reproducibly and specifically detected RNA from EHDV-1 and EHDV-2 in cell

culture and a variety of biological specimens (Aradaib et al., 1994a, Aradaib et al., 1994b, Aradaib et al., 1995b). The serogroup-specific 387 bp PCR products, visualized on ethidium bromide-stained agarose gel or detected with chemiluminescent hybridization, were obtained from the EHDV-318 RNA sample used in this study. This confirms the ability of the previously described PCR-based assay to detect additional serotypes of EHDV from the African continents using the same serogroup primers. The EHDV PCR assay provides supportive diagnostic method to the lengthy and cumbersome conventional virus isolation procedures.

No amplification product or hybridization signals were detected when the serotype-specific EHDV-1 or EHDV-2 PCR-based detection assays were applied to RNA from EHDV-318. These results confirm the variability of the nucleotide sequences of genome segment 2 (L2) among different serotypes of EHDV serogroup (Aradaib et al., 1995c and Aradaib et al., 1995d).

Infections caused by EHDV-318 in local Sudanese breeds of sheep, goats and cattle are usually inapparent and no evidence of clinical hemorrhagic disease has been reported. However, indirect losses associated with decreased milk production and poor subsequent reproductive performance have greater economic impact than occasional overt disease (Mohammed and Mellor, 1990). Studies on experimental EHDV infection demonstrated that cattle can amplify the virus, as determined by conventional virus isolation (VI) and PCR technology, and will become seropositive as determined by the serotype-specific EHDV cELISA (Aradaib et al., 1994b). Using VI and PCR assay, similar results were obtained during evaluation of EHDV infection in sentinel cattle from the central valley of California (Aradaib et al., 1995e). Thus, cattle can provide virus for insect transmission to more susceptible wild ruminants, such as the white-tailed deer populations, where a fatal disease and high mortality may occur. Further studies are needed to determine the complete nucleotide sequence of L2 of EHDV-318. The L2 nucleotide sequence would be advantageous for serotype-specific identification of EHDV-318 isolates using PCR-based detection assay. Widespread application of the molecular biological techniques described in this study should facilitate rapid detection and epidemiological investigation of EHDV outbreaks among susceptible ruminants in the North America and African continents.

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