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Rapid RT-PCR Detection of Epizootic Hemorrhagic Disease Virus Based on *NS2* Gene Sequence Analysis of EHDV Serotype 2

Afraa T.M. Elata and Imadeldin E. Aradaib
Molecular Biology Laboratory, Department of Clinical Medicine,
Faculty of Veterinary Medicine, University of Khartoum,
P.O. Box 32, Khartoum North, Sudan

Abstract: A Reverse Transcriptase (RT) Polymerase Chain Reaction (RT-PCR) based assay for rapid detection of Epizootic Hemorrhagic Disease Virus (EHDV) in cell culture was developed and evaluated. A pair of primers (EHD1 and EHD2), selected from gene segment 8 (NS2) of EHDV serotype 2 (Ibaraki strain) was used for RT-PCR amplification. The RT-PCR assay resulted in amplification of a 379 bp PCR product from 100 fg RNAs extracted from EHDV RNAs. RNAs from North American EHDV serotypes 1 and 2 and RNAs from Central African EHDV serotypes 4 and 6 and a number of EHDV field isolates, propagated in cell cultures were detected by this RT-PCR based assay. Amplification products were not detected when the RT-PCR was applied to RNAs from, closely related orbiviruses including; Blue Tongue Virus (BTV) serotypes 2 and 10; Sudanese and South African isolates of Palyam serogroup orbivirus or total nucleic acid extracts from uninfected Vero cells. The described EHDV RT-PCR based assay should provide a simple method for rapid detection of EHDV. The assay should also be recommended for inclusion during epidemiological surveys of the disease in susceptible wild ruminants and domestic livestock in the African continent.

Key words: Epidemiology, orbiviruses, epizootic hemorrhagic disease, NS2, RT-PCR, Sudan

INTRODUCTION

Epizootic Hemorrhagic Disease (EHD) virus (EHDV) is a double stranded (ds) RNA orbivirus in the family Reoviridae (Borden *et al*., 1971). EHDV often causes a fatal hemorrhagic infection in white tailed deer (*Odocoileus virginianus*) of North America and Blue Tongue (BT) like disease in cattle (Shope *et al*., 1960; Karstad *et al*., 1961; Hoff and Trainer, 1974; Aradaib *et al*., 1997a; Temizel *et al*., 2009). However, in areas of endemicity, the association of EHDV with clinical hemorrhagic disease in cattle is rare and the infection is typically asymptomatic (Gard *et al*., 1988; Mohammed and Mellor, 1990; Abuelzain *et al*., 1992; Gorman, 1992; Aradaib *et al*., 2005). More recent studies on the virus genome analysis indicated that EHDV serotype 3 (Ib Ar 22619) is identical to EHDV serotype 1 (EHDV-1). Therefore, seven serotypes of EHDV serogroup are currently known to be circulating worldwide (Anthony *et al*., 2009a).

EHDV has a genome composed of 10 dsRNA segments (Borden *et al*., 1971). The genome segments code for the Viral Proteins (VP). About 3 Non Structural (NS) proteins and seven Viral Structural (VP) proteins are incorporated into the double layer protein coat (Hammami and Osburn, 1992). The nonstructural proteins NS1, NS2 and NS3 are coded for by genome segments 6, 8 and 10, respectively (Nel *et al*., 1990; Venter *et al*., 1991; Wilson, 1991, 1994a, b). RNA segments 1, 3, 4, 6, 7, 8 and 10 of EHDV were found to be conserved (Anthony *et al*., 2009a). The major protein of the outer coat, VP2, coded for by genome segment 2 has a variable nucleotide sequences among cognate genes of EHDV serotypes and is associated with serotype specificity and induction of neutralizing antibodies (Wilson *et al*., 1990; Aradaib and Ali, 2004).

In previous studies, RT-PCR assays were developed and evaluated for detection of EHDV serotypes based on nucleotide sequences of different genome segments. EHDV PCR assay based on sequence analysis of genome segment 3 (L3) was described (Harding *et al*., 1996). The non-structural protein 3 (NS3) gene of EHDV-1, coded for by genome segment 10 was targeted for development of a single EHDV PCR amplification with chemiluminescent hybridization (Aradaib *et al*., 1998). Nested PCR was also developed and evaluated for detection of EHDV based on NS1 gene sequence analysis of EHDV-2 (Aradaib *et al*., 1994a, b, 1995a-c, 1997a, b, 2003). Recent studies showed

Corresponding Author: Imadeldin E. Aradaib, Molecular Biology Laboratory, Department of Clinical Medicine, Faculty of Veterinary Medicine, University of Khartoum, P.O. Box 32, Khartoum North, Sudan

that segment 8 of EHDV which codes for nonstructural protein 2 (NS2) is conserved among cognate genes of EHDV serotypes (Anthony *et al*., 2009b). Therefore, it was suggested that a fragment of this gene could be targeted for detection of EHDV RNAs in cell culture using RT-PCR. The development of a simple and rapid diagnostic test for detection of EHDV serotypes would be advantageous for a variety of circumstances including; clinical disease investigation, rapid detection of the virus during an outbreak of the disease among susceptible ruminants as well as monitoring of viral incursion in disease free country or in zone free areas.

In the present study, researchers described a simple RT-PCR assay for rapid detection of Sudanese isolates of EHDV serogroup in cell culture using primers derived from segment 8 which codes for *NS2* gene, of EHDV-2.

MATERIALS AND METHODS

Virus and cells: The Sudanese isolates of EHDV serotype 4 and 6; Palyam virus was recovered from sentinel calve herds at the University of Khartoum farm, Shambat, Khartoum North, Sudan (Mohammed and Mellor, 1990). The South African isolate of palyam virus was obtained from the Animal Health Research Institute, Onderstepoort, South Africa. The viruses were processed as described previously (Aradaib *et al*., 1994a). The North American prototypes serotypes of EHDV serogroup (EHDV-1 and EHDV-2) and the BTV prototype serotypes 2 and 10 were obtained from the Arthropod-Borne Animal Disease Research Laboratory, Laramie, WY).

Five field isolates of EHDV were obtained from the National Veterinary Services Laboratories, USDA, APHIS, Ames, IA and Washington Animal Disease Diagnostic Laboratory, Pullman, WA. The virus serotypes and field isolates were propagated on confluent monolayers of Vero cells. The infectious material was harvested and centrifuged at 1,500× g for 10 min and the pellet was used for extraction of viral dsRNA. Details for the RNA extraction was described previously (Aradaib *et al*., 1998). RNAs were quantified using a spectrophotometer at 260 nm wavelength. RNA extracts were then kept frozen at -20°C till used for PCR amplification. Abotu 5 μL of the nucleic acid were used for RT-PCR amplification.

Primer selection: Selection of the primers was based on nucleotide sequence of *NS2* gene of EHDV-2 (Ibaraki strain) with GeneBank accession number AM745084. Primers (20 mer each) were selected from the published nucleotide sequence of genome segment 8 (NS2) of EHDV-2 and used in the RT-PCR assay (Anthony *et al*., 2009a, b). Primers 1 and 2 (EHD1 and EHD2) were selected for the synthesis of the PCR product. EHD1 included bases 19-38 of the positive sense strand of gene segment 8: (5): AAT GGA GCA AAA GCA AAG GA. EHD2 included bases 378-397 of the complementary strand (5): GCC CAT CCC CTT TGA TAT TT. EHDV PCR using primers EHD1 and EHD2 would result in a 379 bp product.

All 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.

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR): A single tube RT-PCR assay was carried out for EHDV RNAs amplification. The RT-PCR protocol used in this study was basically as described previously. Briefly, a standard 50 μL reaction mixture contained in final concentration of 1×enzyme mix reaction buffer, 5.0 μL of 10 mM dNTP mix, 5.0 μL of 25 mM $MgCl_2$, 5.0 U enzyme mix, 2.0 μL of 20 picomole of each primers (EHD1 and EHD2), 5.0 μL of target RNA were used. The total volume was brought to 50.0 μL using RNase free water. Target genes were amplified in low-profile 0.2 mL tube (MJ Research, California, USA). BTV and Palyam dsRNA templates were used as negative controls. All PCR amplifications were carried out at a 50 μL volume per tube.

The thermal cycling profiles were started with a 30 min incubation at 50°C for reverse transcription of the EHDV RNA templates into cDNA copies. The PCR tubes were then incubated at 95°C for 15 min to destroy the excess amount of RT enzyme and to activate the Taq DNA polymerase. The cDNA in the PCR tubes were subjected to 40 cycles of denaturation at 95°C for 1 min, annealing at 56°C for 30 sec and extension at 72°C for 45 sec and a final incubation at 72°C for 10 min. Thermal profiles were performed on a Techne PHC-2 thermal cycler (Techne, Princeton, NJ).

Following amplification, 12 μL from each PCR amplification product were loaded onto 2% agarose gel and electrophoresed at 80 V for 1 h. The gels were stained with ethidium bromide and UV light source was used to visualize the specific 379 bp EHDV PCR products.

RESULTS AND DISCUSSION

The EHDV PCR based assay afforded sensitive and specific detection of EHDV serotypes used in this study. The specific 379 bp PCR product was visualized on ethidium bromide-stained gel from ≥100 fg RNA of United States EHDV prototype serotype 2 (Fig. 1). The 379 bp specific PCR products were detected from 1.0 pg of EHDV

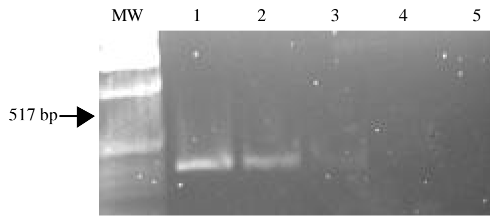

Fig. 1: Sensitivity of the RT-PCR for detection of epizootic hemorrhagic disease virus serotype 2 (EHDV-2). Visualization of the 379 bp specific EHDV PCR product on ethidium bromide-stained agarose gel from EHDV RNA. Lane MW: molecular weight marker; lanes 1-4: (EHDV-2) 10 pg, 1.0 pg, 100 fg and 10 fg, respectively. Lane 5: vero cells total nucleic acid extract

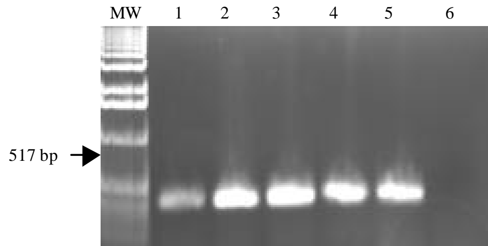

Fig. 2: A visualization of the 379 bp specific-EHDV PCR product on ethidium bromide-stained agarose gel from 1.0 pg of RNA of five different EHDV field isolates. Lane MW: molecular weight marker; Lane 1: EHDV-1; Lane 2: EHDV-2; Lane 3: EHDV-2 ; Lane 4: EHDV-4; Lane 5: EHDV-6; Lane 6: non infected vero cells total nucleic acid extracts

RNA target extracted from North American and African field isolates of EHDV serotypes 1, 2, 4 and 6 (Fig. 2). The specificity studies indicated that the amount of 1.0 pg RNA from closely related orbiviruses including BTV serotypes 2 and 10; Sudanese and South African isolates of Palyam serogroup orbiviruses or total nucleic acid extracts from uninfected Vero cells failed to demonstrate the specific 379 bp PCR products using the EHDV RT-PCR assay (Fig. 3). All EHDV isolates which were PCR positive were also EHDV positive by conventional virus isolation and serotyping. In Sudan EHDV serotype 4 and EHDV-318 are enzootic. EHDV cases fatal hemorrhagic disease in North American white-tailed deer populations and clinical disease in dairy cattle (Shope *et al*., 1960; Hoff and Trainer, 1974; Karstad *et al*., 1961; Work *et al*., 1992; Mohammed *et al*., 1996; Aradaib *et al*., 2005; Temizel *et al*., 2009; Wilson *et al*., 2009a). EHDV-318 was 1st isolated from sentinel calves in Sudan (Mohammed

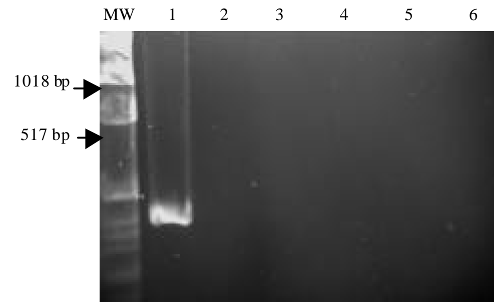

Fig. 3: Specificity of the EHDV RT-PCR for detection of EHDV RNA only. Amplification product was not detected from a concentration of 1.0 ng of BTV RNA from North American BTV prototype viruses; Sudanese and South African isolates of Palyam viruses or total nucleic acid extracts from vero cells. Lane MW: Molecular Weight marker; Lane 1: 1.0 pg EHDV- 2 (Positive control); Lane 2: BTV prototypes serotypes 2; Lane 3: BTV prototypes serotypes 10; Lane 4: Sudanese isolate of palyam virus serogroup; Lane 5: South African isolate of palyam virus serogroup; Lane 6: Vero cells total nucleic acid extractss

and Mellor, 1990). Subsequently, the virus was described in the Sultanate of Oman (Al-Busaidy and Mellor, 1991). The virus was also reported to be a contaminant of genetically-engineered Chinese Hamster Ovary (CHO) cells in Bahrain (Rabenau *et al*., 1993).

Recent studies on genomic analysis of EHDV-318 have identified the virus as EHDV serotype 6 (Anthony *et al*., 2009a). EHDV-6 is currently known to be present in Sudan, Sultanate of Oman, Bahrain, Turkey, United States and countries of the Mediterranean basin including Algeria, Tunisia and Moroco (Temizel *et al*., 2009).

Even in the absence of clinical disease, there is 1961; Work *et al*., 1992; Mohammed *et al*., 1996 restriction on the international trade of livestock and associated germ plasms unless the animals are certified EHDV-free of infection by conventional virus isolation and serology (Aradaib *et al*., 1994a). In addition, the pathological lesions caused by EHDV are undistinguishable from those caused by BTV and hence EHD is of interest to veterinary diagnosticians (Araolaib *et al*., 1994b, 1995a, 1998; Wilson et *al*., 2009a). Moreover, conventional virus isolation and serology are time consuming and cumbersome (Arabdai *et al*., 1994a; Wilson *et al*., 2009b). Therefore, the development of molecular based techniques would be necessary for rapid detection and differentiation of BTV and EHDV. In developing African countries such like Sudan, the described PCR-based

assay should serve as a supportive diagnostic assay to the time consuming and cumbersome conventional virus isolation laboratory procedure. The EHDV field isolates used in this study represented a range of virus serotypes. Of the seven serotypes of the virus recognized worldwide, serotypes 1, 2, 4 and 6 were used in this study. These EHDV serotypes represent the currently known viruses circulating in North America, North and Central Africa.

The EHDV PCR assay would probably detect the remaining serotypes of EHDV serogroup. However, additional research work should be conducted to confirm this assumption. The rapidity, sensitivity and specificity of the PCR assay would greatly facilitate rapid detection of EHDV infection during an outbreak among susceptible ruminants. The PCR assay was a simple procedure that efficiently detected all EHDV field isolates under the stringency condition used in this study.

RNA extraction from infected cell cultures usually takes 30 min and the PCR thermal cycling profiles take 2 h. The electrophoresis of the PCR products on the agarose gels and visualization of results under UV light takes 1 h. Therefore, detection of EHDV in infected cell culture using this PCR assay could be achieved with in the same working day.

It is worth mentioning that this PCR assay for rapid detection of EHDV in cell culture should serve an alternative to conventional virus isolation which is time consuming, tedious, laborious and cumbersome. Further studies are currently under way to improve the sensitivity of the described RT-PCR assay to detect EHDV in experimentally and naturally infected animals and to evaluate its potential as a sensitive and specific diagnostic assay through comparison with current diagnostic test used for detection of EHDV.

CONCLUSION

In this study, the described EHDV RT-PCR assay, using primers derived from genome segment 8 (NS2) of EHDV-2, provides a simple, rapid, sensitive and specific diagnostic method for detection of North American and Central African serotypes of EHDV serogroup in cell culture. The assay should be used as a valuable tool to study the epidemiology of EHDV in susceptible wildlife and domestic livestock in the African continent.

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