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Evaluation of RT-PCR for detection of Sudanese serotypes of epizootic hemorrhagic disease virus serogroup

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ABSTRACT

Epizootic hemorrhagic disease (EHD) is an infectious non-contagious disease of deer and cattle. At least 2 serotypes of EHD virus, designated EHDV-4 and EHDV-318, are enzootic in the Sudan. To facilitate clinical disease investigation and control of the disease a rapid diagnostic assay is urgently needed. A reverse transcriptase (RT) polymerase chain reaction (RT-PCR) protocol, previously reported for detection of the United States EHDV serotypes 1 and 2 ribonucleic acid (RNA) in cell culture and clinical specimens, was evaluated for detection of the Sudanese EHDV serotypes. RNA from Sudanese isolates of EHDV-4 and EHDV-318, propagated in cell cultures, were detected by the described RT-PCR-based assay. The specific 387 bp PCR products were visualized on ethidium bromide-stained agarose gel. Amplification product was not detected when the EHDV RT-PCR-based assay was applied to RNA from Sudanese bluetongue virus (BTV) serotype 4 (BTV-4) or total nucleic acid extracts from uninfected BHK-21 cells. The scientific observations reported in this paper indicated that the previously described EHDV RT-PCR assay could be applied for detection of EHDV infection among the Sudanese susceptible animal populations.

Key words: epizootic hemorrhagic disease, RT-PCR, Sudan

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Introduction

Epizootic hemorrhagic disease virus (EHDV) is a member of the double stranded (ds) RNA *Orbivirus* genus of the family *Reoviridae* (FENNER et al., 1974). EHDV is related to bluetongue virus (BTV) and palyam serogroup Orbiviruses (GARD et al., 1988; ARADAIB et al., 1998c; ARADAIB et al., 1999). The EHDV has a worldwide distribution and exists in at least ten distinct serotypes. In central Sudan, previous studies showed that the incidence of EHDV infection was related to the rainy seasons where the activity of the insect vector, *Culicoides imicola*, is very high (ARADAIB et al., 1999). In the Sudan, four isolates of EHDV, recovered from a sentinel calf herd at the Khartoum University Farm, are known to be enzootic (ARADAIB et al., 1999). The isolates were found to be (EHDV-4) and (EHDV-318), as identified by serum neutralization test (SNT). Isolation of EHDV serotypes was reported in Australia, Asia, Africa and North America (SHOPE et al., 1960; GARD et al., 1988; ARADAIB et al., 1999)

The North American EHDV serotype 1 (EHDV-1) and serotype 2 (EHDV-2) cause an often fatal hemorrhagic disease in white-tailed deer (SHOPE et al., 1960; KARSTAD et al., 1961; METCALF et al., 1991). There is no information available on the impact of EHDV on the wild animals and the small ruminant species in the Sudan, including deer, sheep and goat populations. Infections caused by EHDV in local Sudanese breeds of cattle are thought to be sub-clinical, as no evidence of clinical hemorrhagic disease has been reported. However, reduced production and reproductive performance have a greater economic effect than induction of clinical hemorrhagic disease (reviewed by ARADAIB et al., 1999). Because of unfamiliarity with the ecology and the biology of these viruses, the international movement of livestock between different continents is restricted unless the animals are certified free of infection by conventional virus isolation and serology (OSBURN et al., 1994). This restriction could lead to economic losses for EHDV-endemic countries, such as Sudan, which rely on the sale of livestock for foreign exchange.

Conventional diagnosis of EHDV infection by virus isolation, serology and electron microscopy is time consuming, labour intensive and costly (PEARSON et al., 1992; ARADAIB et al., 1994a; ARADAIB et al., 1995a; ARADAIB et al., 1997; ARADAIB et al., 1999). Molecular diagnostic techniques, including

complementary DNA (cDNA) probes, were developed to improve viral detection methods (WILSON et al., 1990). Genome segment 6 of EHDV-2, which codes for non-structural protein 1 (NS1), was found to be the most highly conserved genome among cognates of EHDV serogroup. Therefore, it was considered of interest to develop a simple, rapid, more sensitive and specific RT-PCR-assay, based on nucleotide sequences of that genome for detection of the United States EHDV serotypes (ARADAIB et al., 1994a). Recently, we described a PCR assay with chemiluminescent hybridization for detection of North American and Central African isolates of EHDV, in cell culture and a variety of tissue samples, based on genome segment 10 of EHDV-1. However, this assay was found to be less sensitive (ARADAIB et al., 1998b).

The objective of the present study was to optimize and evaluate the potential use of the previously described RT-PCR assay (ARADAIB et al., 1994a) for detection of Sudanese EHDV serotypes in cell culture.

Materials and methods

Cell culture, virus propagation and nucleic acid extraction. The four isolates of EHDV present in the Sudan representing (EHDV-4 and EHDV-318) and the Sudanese BTV serotype 4 were used in this study. The viruses were isolated and processed as described previously (ARADAIB and OSBURN, 1994c). All viruses were propagated on confluent monolayers of baby hamster kidney (BHK) cells (ARADAIB et al., 1995a). The infectious material was harvested and centrifuged at 1,500 x g for 30 min and the cell pellet was used for the double stranded (ds) dsRNA extraction as previously described (ARADAIB et al., 1994a).

Primer selection. EHDV Primers were described in detail in a previous report (ARADAIB et al., 1994a). Briefly, primers (20mer each) were selected from the published sequence of genome segment 6 of EHDV-2, which codes for non-structural protein 1 (NS1), and used in the RT-PCR assays. EHDV PCR using primer P1 and P2 would result in a 387 base pair (bp) product.

Polymerase chain reaction. The PCR protocol used in this study was basically as previously described (ARADAIB et al., 1994a). Except that the

Taq DNA polymerase (Perkin-Elmer Cetus, The Perkin Elmer Corporation, Norwalk, CT, USA) was used at a concentration of 2.5 units per reaction. The thermal cycling profiles were as follows: a 2-min incubation at 95 °C, followed by 40 cycles of 95 °C for 1 min, 55 °C for 30 sec and 72 °C for 45 sec, and a final incubation at 62 °C for 10 min. Thermal profiles were performed on a Techne PHC-2 thermal cycler (Techne, Princeton, NJ, USA).

Twenty microliters from each PCR reaction containing amplified product were loaded onto 2% SeaKem agarose (FMC Bioproducts, Rockland, ME, USA) and electrophoresed. The gels were stained with ethidium bromide, washed in double distilled water for 10 minutes, and the specific 387 bp PCR products were visualized under UV light.

Results

The previously described EHDV RT-PCR-based assay afforded sensitive and specific detection of the Sudanese EHDV serotypes and field isolates. The specific 387 bp PCR product was visualized on ethidium bromide-stained gel from 1.0 pg RNA of the Sudanese EHDV serotype 4 (EHDV-4), and EHDV-318 used in this study. The amount of 1.0 ng RNA

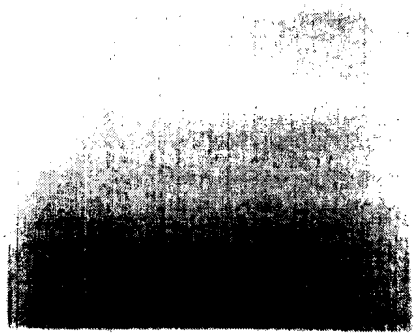

Fig. 1. Detection of the Sudanese isolates of epizootic hemorrhagic disease virus serogroup by RT-PCR. Lane MW: molecular weight marker; Lane 2-4: EHDV-4; Lane 5-6: EHDV-318; Lane 1 and 7: BTV serotype 4 (BTV-4) and total nucleic acid extracted from BHK-21 cells as negative controls, respectively.

Table 1. Specification of myomorphus mammals examined by renoculture and microscopic agglutination acording to the trapping area with corresponding results

from BTV serotypes 4, and total nucleic acid extracts from uninfected BHK-21 cells failed to demonstrate the specific 387-bp PCR products (Fig. 1). All EHDV isolates, which were EHDV-positive by conventional virus isolation, were also EHDV RT-PCR positive.

Discussion

The four EHDV field isolates used in this study represented a range of topotypes viruses, isolated from sentinel calves and the insect vector (*Culicoides imicola*) in Khartoum, Um benein and Nyala regions of the Sudan.

The sensitivity studies of the described RT-PCR protocol are comparable to or even more sensitive than the conventional virus isolation method. The specificity studies indicated that the specific 387 bp PCR product was not amplified from a relatively high concentration of 1.0 ng of RNA from Sudanese BTV-4 or total nucleic acid extracts from non-infected BHK-21 cell controls under the same stringency conditions described in this study.

Despite the small sample size used in this study, which represents the four Sudanese EHDV isolates, we believe the present investigation is acceptable to validate our previously described EHDV RT-PCR assay as a potential diagnostic assay for detection of Sudanese EHDV isolates recovered from cattle or the insect vectors.

It is worth mentioning that this RT-PCR is a serogroup-specific assay, which could be used to detect EHDV serogroup members only. For the last 10 years the only available method for identification of EHDV serotypes has been by the conventional serum neutralization test (PEARSON, 1992; ARADAIB et al., 1994b; ARADAIB et al., 1995a; ARADAIB, 1999). RT-PCR for specific identification of EHDV-2 (ARADAIB et al., 1995b) and serotype-specific identification of EHDV-1 (ARADAIB et al., 1995c) were previously described. Recently, simultaneous serogroup-specific detection and serotype-specific identification of North American serotypes of EHDV-1 and EHDV-2 using RT-PCR was described (ARADAIB et al., 1998a). Subsequently, a multiplex RT-PCR for simultaneous detection and differentiation of EHDV and BTV serogroup members was also described (ARADAIB, 1999). RT-PCR assay for specific identification of the Sudanese

EHDV serotypes is yet to be developed. The previously described RT-PCR assays were successfully applied on a practical scale to evaluate EHDV infection in sentinel cattle from the San Joaquin Valley of California (ARADAIB et al., 2002).

EHDV genome segment 2, which codes for VP2, is associated with serotype specificity and neutralizing antibodies (MECHAM and DEAN, 1988; HUISMANS et al., 1979). Analysis of the nucleotide sequence of VP2 genome would be useful to study the genetic diversity among the Sudanese EHDV isolates. In addition, a fragment of VP2 nucleotide sequences could be targeted to design EHDV specific primers. These primers could then be used in RT-PCR for specific identification of the Sudanese EHDV serotypes. Further studies are in progress to determine the capability of the RT-PCR technology to identify the Sudanese EHDV serotypes, and to evaluate its potential as a sensitive and specific diagnostic assay through comparison with conventional methods used for identification of EHDV infection in the Sudan.

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SAŽETAK

Epizootska hemoragijska bolest je zarazna nekontagiozna bolest jelena i goveda. U Sudanu se enzootski javljaju najmanje 2 serotipa virusa te bolesti i to EHDV-4 i EHDV-318. Za brzinu pouzdanu dijagnostiku i kontrolu potrebne su brze suvremene metode. Za dokaz serotipova izdvojenih u Sudanu vrednovana je lančana reakcija polimerazom uz predhodnu reverznu transkripciju (RT-PCR), opisana u SAD-u za dokaz RNA serotipova 1 i 2 virusa epizootske hemoragijske bolesti u staničnoj kulturi i kliničkom materijalu. RNA sudanskih izolata EHDV-4 i EHDV-318 umnoženih u staničnoj kulturi dokazana je tom metodom. Specifični proizvodi PCR-a od 387 parova baza dokazani su u agaroznom gelu pomoću bojanja etidijevim bromidom. Umnažanje specifičnog slijeda nije dokazano kad je kao izvor nukleinske kiseline rabljen serotip 4 virusa bolesti plavog jezika ili ekstrakti nukleinske kiseline iz neinficirane stanične kulture BHK-21. Na temelju dobivenih rezultata zaključuje se da je prije opisani test u potpunosti prikladan i za određivanje sudanskih serotipova virusa epizootske hemoragijske bolesti.

Ključne riječi: virus epizootske hemoragijske bolesti, RT-PCR, Sudan