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Current status and future prospects of epizootic haemorrhagic disease of deer - a review

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ABSTRACT

Epizootic haemorrhagic disease virus (EHDV), a member of the *Orbivirus* genus in the family *Reoviridae*, is related to bluetongue virus (BTV) and Palyam serogroup of orbiviruses. The EHDV infects domestic and wild ruminants in many parts of the world. Ten serotypes of EHDV designated (EHDV-1 through EHDV-10) are recognized worldwide. Currently, there is little information about the epidemiology of EHDV serogroup of orbiviruses. Cattle are susceptible to EHDV infection, but the disease is usually subclinical. Whereas the disease is fatal in North American white-tailed deer (*Odocoileus virginianus*), no information is available about the disease in African deer populations. Unfamiliarity with the ecology, biology and molecular epidemiology of EHDV serogroup has led to major barriers in international trade of livestock and associated germplasm. The surge of new techniques in cellular immunology and molecular biology should provide an excellent opportunity for understanding the molecular epidemiology of EHDV serogroup members. A vaccine that will provide protective immunity against multiple serotypes, or even a single serotype of EHDV serogroup, is yet to be produced. This article is intended to review the biology, distribution, transmission, pathogenesis, diagnosis, economic importance, import/export regulatory implications, and prevention as well as control measures of these viruses.

Key words: epizootic haemorrhagic disease, epidemiology, molecular diagnosis

1. Structure and distribution of epizootic hemorrhagic disease virus

Epizootic hemorrhagic disease virus (EHDV) is a double-stranded RNA orbivirus in the family *Reoviridae* (BORDEN et al., 1971; FENNER et al., 1974). At least 10 serotypes of EHDV are distributed worldwide (GORMAN, 1992).

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The EHDV prototype virus has a genome composed of 10 double-stranded RNA (dsRNA) segments (HUISMANS et al., 1979; MECHAM and DEAN, 1988). The genome segments code for the viral proteins (VP). Three nonstructural (NS) proteins and seven structural proteins are incorporated into the double layer protein coat (MECHAM and DEAN, 1988). Genome segments 6 and 8 code for the NS1 and NS2, respectively (ARADAIB et al., 1999a). Segment 10 codes for NS3 and NS3a, respectively. The major viral protein (VP) of the outer coat is coded for by genome segment 2 and is associated with serotype specificity and induction of neutralizing antibody (MECHAM and DEAN, 1988). VP1, VP3, VP4, VP5, VP7 and VP6 are coded for by genome segments 1, 3, 4, 5, 7 and 9, respectively. Genomes segments 1, 3, 4, 6, and 8 of EHDV were considered highly conserved with more than 90% homology among cognate genes of other members of the EHDV serogroup (WILSON et al., 1990).

SHOPE et al. (1955) first described EHDV when they reported on a highly fatal hemorrhagic disease among native deer in the United States. They were able to isolate the virus and to identify it as EHDV serotype 1 (New Jersey strain). Subsequently, EHDV serotype 2 was reported in Alberta, Canada (Alberta strain) (SHOPE et al., 1960). Two serotypes designated as EHDV-4 and an untyped isolate designated (EHDV-318) are enzootic in the Sudan (MOHAMMED and MELLOR, 1990). In Australia, 5 serotypes designated as EHDV-5 through EHDV-8 were identified. The fifth serotype was identical to EHDV-2 or Ibaraki virus (ST. GEORGE et al., 1983). In Nigeria, EHDV-3 and EHDV-4 were isolated from *Culicoides spp* (MOORE, 1974). Recently we reported on EHDV- 318, isolated from sentinel calves in Shambat, Khartoum (MOHAMMED et al., 1996). Serological evidence of EHDV infection in goats, deer and cattle is widespread in the Sudan (MOHAMMED and TAYLOR 1987).

2. Transmission

EHDV is arthropod-borne. The virus is transmitted by different species of Culicoides vectors. *Culicoides imicola* is the principal biological vector in the Sudan (ARADAIB et al., 1999a). However, other insects, such as *Culicoides schultzei,* may also serve as biological vectors for EHDV (MOHAMMED et al., 1996). The importance of these vectors and their

distribution are directly related to the distribution of EHDV viruses and, probably, other orbiviruses. 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 imicola* (MOHAMMED and TAYLOR, 1987). Environmental conditions appear to play a crucial role in the distribution of these viruses on any continent, where the activity of the vector is likely to be highest during late summer and early fall (SHOPE et al., 1960). In contrast, the activity of the vector is usually suppressed during the winter season (ARADAIB and ABBAS, 1985). However, transmission of the disease during the winter season is not uncommon (ARADAIB et al., 1994a). The mechanism of overwintering transmission is yet to be explained. Cyclonic winds and warm temperatures determine how far the infection would be transmitted. It has been suggested that EHDV infected semen may also play a role in the transmission of the disease. However, additional research is necessary to confirm this assumption. Another mode of EHDV transmission could be the use of contaminated modified live virus vaccine through contamination of cell cultures, as the virus is known to be a contaminant of Chinese hamster ovary (CHO) cells (RABENAU et al., 1993).

3. Pathogenesis

Very little information is available with regard to the pathogenesis of EHDV serogroup on the African continent. Clinical hemorrhagic disease caused by EHDV in cattle and wild ruminants, particularly the North American white-tailed deer, was reported (SHOPE et al., 1955; SHOPE et al., 1960; FOSGERG et al., 1977; KARSTAD et al., 1961). Experimental EHDV infection in sheep, goats and cattle failed to produce clinical signs of the disease. However, viremia was produced (GIBBS and LAWMAN, 1977; ARADAIB and OSBURN, 1994c).

a. Clinical signs

The clinical signs seen in cattle infected with EHDV were indistinguishable from those seen in cattle infected with BTV virus (KARSTAD et al., 1961). Clinical signs of EHD in white-tailed deer include fever, mucosal congestion, inappetence, drooling, dyspnea, recumbency,

and terminal convulsions. Haemorrhages from the natural body orifices are inconsistent findings. Sudden deaths are usually observed in peracute cases and infected animals die within seven days (OSBURN, 1994; OSBURN et al., 1994).

b. Post-mortem findings

Necropsy findings of peracute cases include yellow gelatinous subcutaneous and intramuscular oedema, pulmonary oedema, straw-coloured transudate in the thoracic pericardium and abdominal cavities, haemorrhages in the subcutis, heart, and fore-stomach. Hemorrhagic enteritis is observed on occasions, renal haemorrhages or degeneration were also seen. Lesions associated with chronic disease include sloughing of the hooves with or without secondary infection, damaged rumen mucosa, oral ulcer and emaciation (SHOPE et al., 1955). In cattle the Ibaraki virus strain of EHDV was originally isolated from cattle in Japan (1959-60) during a large BTV-like epizootic. Mortality was 1.96% (INABA, 1975).

4. Diagnosis

a. Virological techniques

Preparation of blood samples. Heparinized blood samples collected from the jugular vein are used for virus isolation (VI) and detection of virus nucleic acid sequence by PCR. Processing of the blood samples for VI was as described previously (STOTT et al., 1978). Briefly, the blood cells were washed twice with phosphate buffered saline (PBS) containing 100 units penicillin and 200 units mcg streptomycin/ml. The washed blood cells were restored to the original volume with 2 mM Tris buffer pH 8.0 to lyse the blood cells.

Clinical samples. Processing of the tissue samples, including spleen and lung suspensions, for virus isolation (VI) was as described previously (ARADAIB et al., 1994a). Briefly, plasma was discarded and blood cells were washed twice with phosphate buffered saline (PBC) containing 100 units penicillin and 100 units mcg streptomycin/ml. The washed blood cells were restored to the original volume with 2 mM Tris buffer pH 8.0, and blood cells were then disrupted by sonication. The spleen sample was

homogenized 1:10 (W/V) in minimal essential media (MEM), and cells debris was sedimented by centrifugation at 1,500 g for 20 min.

Cell culture. BHK-21 cells or Vero cells are usually used for virus propagation. Minimal essential medium (MEM) containing 100 units penicillin/ml and 100 mg streptomycin/ml, 10% tryptose phosphate broth and 10% foetal bovine serum (FBS) that has been heat inactivated at 56° C for 30 min are usually supplemented. Cell cultures were then incubated at 37 °C in a humidified incubator with 5.0% carbon dioxide tension until confluent monolayers were obtained (usually 2-3 days).

*Virus isolation and identification.*Various laboratories use different methods for isolation of EHDV, including intravenous (IV) inoculation of embryonated chicken eggs (ECE) or isolation on cell lines (ARADAIB et al., 1994b; ARADAIB et al., 1994c; GARD et al., 1988; PEARSON et al., 1992; ARADAIB et al., 1995a). The VI technique is laborious, expensive and time consuming and a final result required 2-4 weeks to obtain.

The BHK-21 cell monolayers were inoculated with lysed blood or spleen homogenate diluted 1:10 in MEM. After incubation at 37 °C for 1 hour the inoculated cell cultures were supplemented with MEM, 2% tryptose phosphate broth, and 2% FBS. The cell cultures were again incubated at 37 °C and observed daily until cytopathic effect was 80% complete. Cultures with no cytopathic effect were blind passaged. All cytopathic agents were identified by a plaque inhibition assay (ARADAIB et al., 1995a). Six eleven-day-old embryonated chicken eggs (ECE) were each inoculated intravenously with 0.1 ml of diluted lysed blood, received from suspect animals with clinical hemorrhagic disease. Inoculated ECE were incubated at 33 °C for 7 days. Eggs were candled daily and dead embryos were removed. Dead and live embryos were harvested, macerated, sonicated, diluted 1:1000 in PBS and used for a second passage. In our laboratory we observed that the use of trypsin at a concentration of 2ug/ml increased the sensitivity of the BHK-21 cell monolayer for isolation of the virus (ARADAIB et al., 1994b).

The serotype-specific identification of the virus is usually based on serum neutralization test using known reference antiserum (PEARSON et al., 1992). Plaque inhibition test could also be used as a supportive diagnostic test (STOTT et al., 1978).

b. Serological techniques

Serology does not necessarily identify an active infection. However, it is useful for determining past infection in a seroepidemiological survey (ARADAIB and ABBAS, 1985). Several serodiagnostic techniques have been validated for the diagnosis of orbiviruses. The main bulk of the work to date has been carried out on bluetongue, a closely related orbivirus. The serological techniques applied for diagnosis of EHDV include agar gel immunodiffusion (AGID), the complement fixation test (CFT) and the indirect labelled antibody technique, such as the indirect immunofluorescence antibody technique (IFAT), the indirect enzyme linked immunosorbent assay (ELISA), the competitive ELISA (cELISA) using monoclonal antibody (Mab), and immunoblotting for the viral proteins, such as Western blots.

Agar-gel immunodiffusion test (AGID). The method adopted was basically that described by ARADAIB et al. (1994b). Precipitin lines were also observed with sera from calves experimentally infected with EHDV serotypes 1 and 2 (ARADAIB et al., 1994b). Cross-reactions in the AGID between different serotypes of the Orbivirus serogroup were also observed, but no precipitin lines were observed with sera from non infected control calves.

Complement fixation test (CFT). The CFT requires the use of sensitized RBCs of sheep. A positive result is indicated by absence of RBCS haemolysis due to fixation of the complement by the antigen antibody reaction. Haemolysis of the RBCs indicates a negative test. The test is sensitive and has a very high specificity in the diagnosis of EHDV infection. However, the test is a complicated, highly delicate technique and is not suitable for field operation (PEARSON et al., 1992).

Enzyme-linked immunosorbent assay (ELISA). The potential of ELISA for diagnosis of EHDV infections has been investigated and ELISA was found to be more sensitive than earlier serological tests (MOHAMMED and MELLOR, 1990). ELISA is more likely to be used in developing countries, such as on the African continent, since minimal laboratory equipment is required. In addition, ELISA could be used as a superior diagnostic alternative to other serological tests because it is a simple, rapid, inexpensive and sensitive method. One of the major problems in the

application of ELISA for EHDV diagnosis is the availability of suitable and specific antigens that could be used to avoid, or to minimize, false positive results due to cross-reactions. Most investigators have used crude or partially purified antigens (MOHAMMED and MELLOR, 1990). Unfortunately, crude antigen yielded no diagnostic value for EHDV serotype-specific identification. Monoclonal antibodies to EHDV were produced and were validated for detection of EHDV serogroup and serotype-specific using competitive ELISA (cELISA) techniques (ARADAIB and OSBURN, 1994c). However, cELISA requires at least 2 weeks for production of EHDV antibodies by the susceptible host. To address these problems, molecular diagnostic techniques have been developed.

c. Molecular diagnostic techniques

For the last twelve years the major thrust of our research has been oriented towards the improvement of existing techniques used for diagnosis of EHDV infection. The development of a rapid, sensitive, specific and inexpensive method for diagnosis of the disease would greatly facilitate clinical disease investigations, epidemiological investigation, treatment of the infected animals, and would enhance vaccination and control programs (WILSON et al., 1992; ARADAIB et al., 1994a; ARADAIB et al., 1994b; ARADAIB et al., 2003).

Sub-unit core and outer coat proteins. Recent techniques in immunology have made possible the development of monoclonal antibody (Mab) directed against specific-EHDV epitope. We were able to use this Mab in competitive ELISA (cELISA) for detection and specific identification of EHDV-1 and EHDV-2 (ARADAIB et al., 1994b).

Immunoblotting technique (Western blotting). This technique has been recently introduced for detection of viral protein 2 of bluetongue virus recombinant vaccine using baculovirus expression system. The viral proteins are run in polyacrylamide gel and transferred to nitrocellulose papers or nylon (Zeta) membranes. The blotted proteins are then incubated with EHDV antisera at room temperature and then with labelled antibody conjugate at room temperature. Finally, a substrate is added for visualization of the antigen-antibody reaction at the expected molecular weight of the protein using molecular weight marker. The Western blot is highly sensitive and specific. The technique is useful to study the kinetics of immune

responses of animals to Orbiviruses vaccines as well as correlation between antibody production and protection (ARADAIB et al., 2000a).

Despite their advantage in detecting infected individuals the serological techniques mentioned previously are complicated by cross-reactions between EHDV serogroups and other members of the *Orbivirus* genus. Although the use of monoclonal antibodies (Mab) in competitive ELISA (cELISA) technique has improved sensitivity and specificity at the serotypes level (ARADAIB et al., 1995b), this technique is applicable only to blood samples (serum) and requires at least 14 days post-infection for the production of anti-EHDV antibodies by the susceptible host (ARADAIB et al., 1994a).

Polyacrylamide gel electrophoresis (PAGE). The dsRNA is usually used at a concentration of 100 nanograms and the PAGE is used at a concentration of 10 % (HAMMAMI and OSBURN, 1992). Using the PAGE, the EHDV-318 ribonucleic acid showed 10 dsRNA segments. EHDV-318, EHDV-1 and EHDV-2 dsRNA migrated to the same length but with different electropherotic patterns. Nucleic acid analysis of EHDV-318 by PAGE showed 10 distinct dsRNA genome segments that represent the characteristic pattern of Orbiviruses. Tentative diagnosis of an EHDV infection could be made using the PAGE technique. However, sufficient electrophoretic variation exists in field isolates of EHDV to present different electrophoretic patterns between serotypes. Therefore, different serotypes of EHDV serogroup may have the same electropherotype and different Orbiviruses may have indistinguishable electropherotypes (MOHAMMED et al., 1999). Thus, electrophoretic patterns themselves have no definitive diagnostic value for detection of EHDV infection. In a previous study the genomes of EHDV-318 (Africa), EHDV-1 and EHDV-2 (North America) serotypes migrated to the same length despite the different electropherotypic patterns of their dsRNA. This finding suggests that it would be interesting to compare the lengths of EHDV electropherotypes from North America or Africa with their Asian and Australian counterparts. The difference in length between electropherotypes as determined by their migration in PAGE, if any, could then be used as a valuable tool to study the epidemiology of EHDV from different continents (HAMMAMI and OSBURN, 1992).

Nucleic acid hybridization and amplification technology. This requires extraction of the ds RNA from cell culture. Details for total nucleic acid extraction has been described previously (ARADAIB et al., 1994a).

RNA probes. The extracted viral RNA is usually run in PAGE and the RNA segments will then be identified according to their electrophoretic migration on the gel. The RNA genome segment of interest will be eluded from the gel, labelled with radioactive material and used as a probe. The nucleotide sequences of the conserved EHDV probe will be specifically hybridized with the corresponding segment of different EHDV serotypes (NEL and HUISMANS, 1990; WILSON et al., 1992). After Northern blot of the RNA these RNA probes could be used to hybridize with the corresponding segment of EHDV serogroup. Because RNA probes are unstable, they are not commonly used in Northern blot hybridization assays.

cDNA Probes. Complementary DNA (cDNA) probes are stable and are more commonly used as probes in Northern blot hybridization of RNA. cDNA probes are used in Southern blot hybridization. The cDNA probe of a particular segment could be used as a fragment of the segment, or as a full-length genome. The cDNA could also be synthesized by cloning in a vector (WILSON et al, 1990). The cloning procedure is tedious, laborious and time consuming. Recently, PCR-generated probes have been described and used for detection of EHDV serogroup (MOHAMMED et al., 1996). 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 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 manufacturer's instructions and used for hybridization of the blotted nucleic acids. Chemiluminecsent hybridization was performed as described by ARADAIB et al. (1994a).

Nucleic acid hybridization (NAH) assay using cDNA probes. For detection of EHDV RNA, dot blot, slot blot and Northern blot hybridization assays were used. However, for detection of cDNA, Southern blot is the

most common procedure. The probe could be labelled with radioactive material such as ^{32}P or ^{125}I (De MATTOS et al., 1989). The nonradiolabelled procedure of the probe obviates the hazardous and cumbersome laboratory procedure of working with radioactive materials. The enzyme labelling procedure of working with non-radiolablled chemiluminescent hybridization assay has been described (ARADAIB et al., 1994a; ARADAIB et al., 1995c; ARADAIB et al., 1995d; ARADAIB et al., 1997a).

Probes derived from segments 6 and 8 of EHDV-2 (Alberta) did not hybridize with BTV dsRNA (WILSON et al., 1990). A probe derived from genome segment 3 of EHDV-1 cross-hybridized with the corresponding segment of EHDV-2, but not with BTV viruses isolated in the United States (WILSON et al., 1990). The best hybridization signal with all EHDV isolates was obtained using a probe derived from genome segment 6 that codes for NS1 (NEL et al., 1990). The greater sensitivity of the NS1 gene-specific probe is ascribed to the fact that its target, the NS1 mRNA, is transcribed more frequently than other viral target mRNAs. In addition, it required only a small number of infected cells to produce a positive hybridization signal than was the case with the other nucleic acid probe.

The gene coding for viral protein 2 (VP2) has been identified, cloned and sequenced (VENTER et al., 1991). Nucleic acid hybridization techniques using Complementary DNA (cDNA) probes have been developed for detection of nucleic acid sequence of EHDV genome segments. Using cDNA probe, the dot blot hybridization technique showed a positive hybridization signal with dsRNA from EHDV serogroup (MOHAMMED et al., 1996; WILSON et al., 1990; WILSON et al., 1992). Use of the peroxidase-labelled cDNA probe obviates the hazardous and cumbersome laboratory procedure of working with radioactive labelling techniques. 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. Because of its safety and rapidity, this technique readily lends itself to use in developing countries for the detection of EHDV in cell culture.

Southern blot hybridization. Southern blot hybridization was performed basically as previously described (ARADAIB et al., 1994a). The probe prepared by PCR was labelled 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 h. The labelled probe was added to the hybridization buffer and the membranes were hybridized overnight. After post hybridization 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. However, one of the disadvantages of dot blot hybridization is that the technique is incapable of direct detection of EHDV RNA in clinical samples from infected animals. Hence, a prior amplification step by PCR technology is deemed necessary.

Polymerase chain reaction (PCR). Application of polymerase chain reaction (PCR) has proliferated because of its simplicity, rapidity, reliability, reproducibility, sensitivity and specificity for monitoring of microorganisms. PCR proved satisfactory to replace the immunological and DNA hybridization methods using cDNA probes.

Reverse transcriptase (RT- PCR) for EHDV serogroup-specific detection. EHDV RT-PCR has been successfully applied for detection of RNA viruses by addition of a complementary DNA (cDNA) synthesis step using reverse transcriptase (RT) enzyme, before cyclic amplification by PCR. RT- PCR-based assay for detection of EHDV serogroup in cell culture and clinical samples, using primers derived from NS1 genome sequence analysis of EHDV-2 Alberta strain, has been described (ARADAIB et al., 1994a).

This EHDV RT-PCR was compared with different virus isolation (VI) procedures from a variety of tissue samples. Results indicated that the sensitivity of the previously described EHDV-PCR assay is comparable to, or more sensitive than, the VI method in BHK-21 cell culture or ECE. In addition, the EHDV RT-PCR assay could provide a superior diagnostic alternative to replace the current cumbersome and time-consuming virus isolation procedures (ARADAIB et al., 1995a). Serogrouping and topotyping of United States and Sudanese isolates of EHDV were also compared using EHDV RT-PCR (ARADAIB et al., 1997a).

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., 1994c). The serogroup-specific 387 bp PCR products, visualized on ethidium bromide-stained agarose gel or detected with chemiluminescent hybridization, were obtained from the EHDV RNA sample used in this study. This confirms the ability of the PCR assay to detect new serotypes of EHDV from different continents using the same serogroup primers. The EHDV PCR assay provides an attractive diagnostic alternative to the lengthy and cumbersome conventional virus isolation procedures. The probe derived from segment 10 0f EHDV-1 hybridized with cognates of EHDV serotypes 1, 2, 4 and 318 but not with those of BTV (ARADAIB et al., 1998a).

Studies on experimental EHDV infection demonstrated that cattle could 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 San Joaquin l Valley, California. Thus, cattle can provide virus for insect transmission to more susceptible wild ruminants, such as white-tailed deer populations, where fatal disease and high mortality may occur.

Reverse transcriptase (RT-PCR) for EHDV serotype-specific detection. Specific identification of EHDV serotype 1 (EHDV-1), in cell culture or tissue samples, was described using RT-PCR-based assay (ARADAIB et al., 1995d). A similar study was conducted to demonstrate specific identification of EHDV-2 in cell culture and clinical samples (ARADAIB et al., 1995c). These RT-PCR assays were based on nucleotide sequences of genome segment 2, which codes for viral structural protein 2 (VP2). This nucleotide sequence of the genome was found to be variable among cognate genes of different serotypes of EHDV serogroup. No cross- amplification product or hybridization signals were detected between serotype-specific EHDV-1 and EHDV-2 PCR-based detection assays. In addition, using RNA from EHDV-318, the described serotype-specific (EDDV-1) or (EHDV-2) assays failed to produce the specific PCR products. This result confirms the variability of the nucleotide sequences of genome segment 2 (L2) among

cognates of different EHDV serotypes. 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 using RT-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 North America and on other continents.

Nested polymerase chain reaction. In the nested PCR, two pairs of oligonucleotides primers are required. The PCR reaction is carried out in 2 amplification steps. The first pair of primers (outer primers) is used to amplify a specific PCR product. The second pair of nested (internal) primers is designed internal to the annealing sites of the outer primers to amplify a specific PCR product, which is shorter than that produced by the first pair of primers.

The second amplification step using the nested primers is necessary to confirm the specificity of the first amplified product and to increase the sensitivity of the PCR-based assay.

The use of nested PCR obviates the hazardous and cumbersome radioactive laboratory procedures of working with ^{32}P or ^{33}P (ARADAIB et al., 1998b).

Multiplex polymerase chain reaction. In this type of PCR different primer pairs derived from different EHDV serotypes are used in a single-tube PCR amplification. A multiplex RT-PCR for simultaneous serogroup-specific detection and serotype-specific identification of North American EHDV-1 and EHDV-2 has recently been reported (ARADAIB et al., 1998c). A multiplex RT-PCR-based assay, for simultaneous detection and differentiation of BTV and EHDV, in cell culture was also described (ARADAIB et al., 1999a; ARADAIB et al., 1999b; ARADAIB et al., 2003). Primers designed from NS3 genome of BTV-10 and NS1 genome of EHDV-2, reproducibly and specifically detected BTV and EHDV RNAs in infected cell cultures. A similar study was conducted to differentiate between North American BTV and EHDV serotypes in clinical samples using multiplex RT-PCR amplification technology. Primers were designed from NS1 genome of BTV-17 and NS1 of EHDV-1 (ARADAIB et al., 2003). The

multiplex RT-PCR assay was a simple procedure that utilized a single-tube PCR amplification in which EHDV and BTV primers were used simultaneously

Simultaneous detection and differentiation of Orbivirus serogroup will simplify the assay, save time and, above all, save on cost, because each clinical sample will be tested once instead of testing the suspected samples individually. Because of its rapidity, sensitivity and specificity, the multiplex RT-PCR assay would be advantageous in epidemiological investigations where field isolates can be tested to determine the prevalence and frequency of Orbivirus infections in susceptible animal populations, and can also be used for evidence of viral incursion in a particular geographical region.

The multiplex RT-PCR assay could be used for export regulation to certify animals free from Orbivirus infections. Because of cross contamination, extraction of dsRNAs from different members of the Orbivirus serogroup should be carried out in separate rooms. Aerosol-resistant tips should be used to minimize contamination. Negative and positive controls should be included in each PCR amplification to estimate the lower limit of specificity and the higher limit of sensitivity. A definitive diagnosis of an infected clinical sample using the EHDV PCR-based detection assay with chemiluminescent hybridization could be obtained within 2-3 days (ARADAIB et al., 1994a) and within the same working day using a nested PCR-based assay (ARADAIB et al., 1998b). It is worth mentioning that conventional virus isolation procedures will remain important for recovery of an infectious virus and for understanding the biology, genetic diversity and the epidemiology of their diseases.

5. Significance and regulatory implications

The precipitation that EHDV serotypes are transmitted from one country to another by animals and that the disease cause widespread death losses has led to the Office International des Epizooties (OIE) to include them in list A, implying that they are among some of the most important transmissible infectious diseases in the world. EHDV infection is of significance to veterinary diagnosticians as well as to animal industries as it imposes restrictions on the international trade of livestock, animal-derived

products and associated germplasm (BRANNIAN et al., 1983; HOFF and TRAINER; 1974; WORK et al., 1992; OSBURN et al., 1994). Also, the economic losses attributed to the often subclinical disease are difficult to estimate (ARADAIB et al., 1997b). In the Sudan, isolate 318 (EHDV-318) was reported to be associated with reduced productivity and reproductive efficiency in sentinel cattle at the Khartoum University Farm, Shambat (MOHAMMED et al., 1996). In addition, EHDV-318 was reported to be a contaminant of genetically engineered Chinese hamster ovary (CHO) cells in Bahrain (RABENAU et al., 1993). Infections caused by EHDV-318 in local Sudanese breeds of sheep and goats are usually unapparent and no evidence of clinical hemorrhagic disease has been reported. However, indirect losses associated with decreased milk production and poor subsequent reproductive performance have a greater economic impact than occasional overt disease (MOHAMMED and TAYLOR, 1987; ARADAIB et al., 2000).

The sale of livestock and associated germplasm may be affected in the international market, unless the animals are certified free from infection by conventional virus isolation or serology (OSBURN et al., 1994).

Although more than 48 years have elapsed since the discovery of EHDV by SHOPE et al. (1955), only the rudiments of its epidemiology are known. It is well documented that EHDV infection is the most serious unresolved veterinary problem in North American native deer. Epizootics of clinical haemorrhagic disease among Sudanese deer was reported several times during late summer and early fall (BADAWI, unpublished data). The causative viral agent was identified as a BTV isolate. However, identification was based on agar gel immunodiffusion (AGID) test. It is well documented that the AGID test is a group-specific test and cross-reaction is likely to occur between different members of the orbivirus serogroup, including EHDV and Palyam serogroup Orbiviruses (MOHAMMED et al., 1999). Therefore, it was not known exactly whether those isolates were really BTV or BTV-related orbivirus.

6. Prevention and control measures

Prevention and control of EHDV serotypes are dictated by climatic conditions. Control of the disease by destruction of vector species of the insect is expensive, extremely difficult and is not a valid option in large

countries, especially when it is recognized that reintroduction of the vectors via the wind from surrounding territories is likely to occur (MOHAMMED and TAYLOR, 1987). The control measures should be aimed at minimizing contact between susceptible animals and vectors, particularly during EHDV-seasons. The use of insect-secured houses and insect repellents for susceptible ruminants should be considered during the flight time of the vector. There is no vaccine for immunization of susceptible animal populations against EHDV infections. Because there are ten distinct EHDV serotypes, the development of an effective vaccine would be extremely difficult, if not impossible. It should be taken into consideration that if a modified live virus is to be used as a vaccine, the modified virus will spread to more susceptible animals via the *Culicoides* vectors. In addition, the use of a modified vaccine, containing two or more serotypes of EHDV, may enable these viruses to reassert in nature, giving rise to a new infectious form. The segmented nature of the viral genome and the reassertment of the virus in vivo have led to the development of vaccine markers. A innovate sub-unit vaccine for BTV was developed in the baculovirus expression system. The sub-unit vaccine induced protective immunity. However, the vaccine requires the use of adjuvants to provide long-lasting protective immunization (OSBURN, 1994). No similar studies were conducted to provide information with regard to vaccination against EHDV infection. A broad-spectrum EHDV vaccine that can protect against EHDV serotypes has yet to be produced.

Conclusion

Epizootic hemorrhagic disease virus (EHDV) may cause fatal hemorrhagic infection in North American white-tailed deer and subclinical infection in cattle. Antibodies to EHDV have been detected in sera from cattle, sheep, goat and deer. Clinical hemorrhagic diseases were frequently observed among deer populations in Sudan and Saudi Arabia, which is suggested to be caused by EHDV. However, virus isolation attempts were largely unsuccessful. Evaluation of EHDV infection in sentinel cattle herds, by routine surveillance for virus infection and associated vector transmission of EHDV, is the backbone of epidemiological studies of EHDV in a given geographical location. In the absence of clinical

hemorrhagic disease, sentinel cattle herds provide the most effective approach for monitoring animal health and incursion of viral infection, including recovery of new EHDV serotypes to a particular location.

The application of bioengineering to study the molecular epidemiology of EHDV should facilitate detection of active EHDV infections, thus enhance herd health monitoring. Vaccination and control programs against EHDV infection could be improved by development and administration of attenuated live virus vaccines during the period of the year in which the insect vectors are inactive. In addition, the use of a recombinant vaccine in the baculovirus expressing sub-unit core and outer coat proteins (without nucleic acids) should be developed to limit the level of transmission and the extent of the pathology of the disease.

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SAŽETAK

Virus epizootske hemoragijske bolesti jelena (VEHB), kao pripadnik roda *Orbivirus*, porodice *Reoviridae*, srodan je virusu bolesti plavog jezika i serološkoj skupini Palyam orbivirusa. VEHB zarazan je za domaće i divlje preživače u mnogim dijelovima svijeta. Diljem svijeta poznato je 10 serotipova VEHB-a, označenih VEHB-1 do VEHB-10. Zasad postoji malo podataka o epizootiologiji serološke skupine VEHB orbivirusa. Goveda su prijemljiva za infekciju VEHB-om, ali bolest je obično supklinička. Bolest je smrtonosna za bjelorepog jelena *(Odocoileus virginianus*) u Sjevernoj Americi, ali nema nikakvih podataka o njoj u populaciji afričkih jelena. Nepoznavanje ekologije, biologije i molekularne epidemiologije serološke skupine VEHB dovelo je do velikih prepreka u međunarodnoj trgovini stokom. Uvođenje novih tehnika u staničnu imunologiju i molekularnu biologiju pružit će mogućnost za razumijevanje molekularne epidemiologije pripadnika serološke skupine VEHB. Vakcina koja bi pružila zaštitnu imunost protiv više serotipova ili samo jednog serotipa serološke skupine VEHB još nije proizvedena. Članak prikazuje biologiju, proširenost, prenošenje, patogenezu, dijagnostiku, gospodarsko značenje, reguliranje uvoza i izvoza te mjere za kontrolu i sprečavanje bolesti.

Ključne riječi: virus epizootske hemoragijske bolesti jelena, epidemiologija, molekularna biologija