Epizootic Hemorrhagic Disease Virus Type 6: Disease, RNAemia and Abortions - The Israeli Experience

Natalia Golender1, Yevgeny Khinich1, Michael Bernstein2, Nir Edery3, Marisol Guini1, Evgeny Eyngor1 and Velizar Bumbarov*1
1Division of Virology, Kimron Veterinary Institute, Beit Dagan 50250, Israel
2Division of Bacteriology, Kimron Veterinary Institute, Beit Dagan 50250, Israel
3Division of Pathology, Kimron Veterinary Institute, Beit Dagan 50250, Israel

Abstract

Epizootic Hemorrhagic Disease (EHD) is a non-contagious viral disease affecting mostly white-tailed deer and cattle. It can cause fever, hemorrhages, excessive salivation, agalactia, loss of body weight, abortion and occasionally death. A large outbreak of epizootic hemorrhagic disease virus serotype 6 (EHDV-6) was identified in several Israeli cattle farms towards the end of 2015. In this study, we examined the persistence of EHDV RNAemia in whole blood (RNAmia) from six naturally infected sentinel calves. RNAmia duration was estimated based on real-time reverse transcription polymerase chain reaction (RT-qPCR) of whole blood samples. Our results indicate that EHDV RNAmia lasted for approximately four months from the presumed date of the initial natural infection. Additionally, based on the data of PCR-positive field samples from sick animals and aborted cattle fetuses, along with isolated viruses from samples taken at the same period of time as a routine diagnostic procedure, we conclude that the outbreak took place between late September and November 2015. Analysis of abortion cases throughout this period (all aborted fetuses and placentas tested for abortogenic pathogens), suggests that EHDV-6 was involved in the part of abortion cases in this cattle population.

Introduction

Epizootic Hemorrhagic Disease (EHD) is an infectious, non-contagious viral disease of ruminants transmitted by insects of the genus Culicoides. The virus belongs to the genus Orbivirus within the family Reoviridae. At least seven serotypes are currently recognized (http://wahis2-devt.oie.int/fileadmin/Home/fr/Health_standards/table-m/2.01.04b_EHD.pdf).

EHDV has been identified in Japan, Africa, North America, Australia and Mediterranean Basin. EHD in cattle was first described in Japan (“Ibaraki disease” or EHDV-2; Omori T.et al., 1969), but the 2006 outbreak of EHDV-7 in Israel [1]. EHDV-6 in several countries of Mediterranean Basin [2] and the 2012 outbreak in the US (EHDV-2, EHDV-6 and EHDV-1) has increased the concerns of EHDV affecting cattle [3]. In 2015 EHDV-6 outbreaks in cattle were recorded in Tunisia and Israel (http://www.promedmail.org/post/3685159). Clinical signs observed in Israel, were characterized by a drop in milk production, weakness, excessive salivation, lameness, recumbency, pyrexia, mild erythema of nasal and oral mucosas, weight loss, abortion and occasional death of animals [4].

Orbiviruses such as Bluetongue virus (BT) can circulate in the host for a prolonged time after the clinical signs have resolved. RNAemia can continue for a period of 25 months [5]. However, the data concerning EHDV circulation is scarce, and little is known regarding the duration of RNAemia or sub-clinical infection in cattle. Additionally, EHDV has never been reported as identified or isolated from fetuses. In this study, we monitored the duration of RNAemia in naturally infected calves. Additionally, we estimated duration of the EHDV outbreak in Israel in 2015.

Materials and Methods

RNA extraction, RT-qPCR and conventional RT-PCRs

RNA extractions from field samples (EDTA-anticoagulated whole blood, lung, intestine, liver, heart, kidney, placenta and brain) and from ECE virus isolates followed by pan-EHDV RT-qPCR and conventional RT-PCR were performed as described previously [4,6].

In order to identify Simbu group viruses in bovine aborted fetuses, the in-house Simbu serogroup-specific Uni-S conventional RT-PCR was performed using a One-Step RT-PCR kit (Qiagen, Hilden, Germany).

Gel purification

PCR products resulting from positive samples were consequently purified using the MEGA quick-spin™ Total Fragment DNA...
Purification Kit (iNtRON Biotechnology, Gyeonggi-do, South Korea) and analyzed on the ABI 3730xl DNA Analyzer, Hylabs, Rehovot, Israel.

Monitoring of RNAmia and antibody appearance in naturally infected calves

Whole blood and serum samples from six randomly-selected calves (approximately 8 months old at the beginning of monitoring) were sampled monthly from July 2015 to April 2016 and consequently tested by RT-qPCR for presence of EHDV. The serum samples were tested for the presence of EHDV antibodies using LSIVet Ruminant EHDV – Serum ELISA Ab, Lissieu, France.

Virus isolation

EHDV was isolated on Embryonated Chicken Eggs (ECE) and Cell Cultures (CC) as described by [7-9].

BVD Ag ELISA tests

All internal organs from aborted fetuses (brain, lung, spleen, liver, intestine, heart and kidney) and placentas were homogenized in PBS (pH 7.2) to a final concentration of 1:10. The supernatant from these centrifuged samples was used for further BVD antigen ELISA testing by the IDEXX BVVD Ag/Serum Plus Test, Liebefeld-Bern, Switzerland, according to the manufacturer’s protocol.

Bacteriological testing

Internal organs from aborted fetuses and placentas were transferred after necropsy for bacteriological testing. Tissue samples, including abomasal contents, were streaked onto MacConkey, blood, nutrient, Skirrow (Campylobacter) and Brucella (selective trypticase soya) agar plates. Testing for Mycoplasma spp. was performed by inoculation of abomasal contents into Fry’s mycoplasma broth (Freundt, 1983) and platedonto mycoplasma agar. Placental, lung and abomasal smears were stained by Stamp’s modified Ziehl Neelsen method and with an FITC monoclonal antibody for Chlamydia spp. (Cellabs, Australia). Abomasal contents were inoculated into tetra-thionate broth for Salmonella enrichment which was streaked onto MacConkey and Brilliant Green agar after 24 hours incubation. All cultures excluding Salmonella were incubated in a 5% carbon dioxide atmosphere.

MAT for Leptospira Spp.

In order to detect exposure to Leptospira spp., the Microscopic Agglutination Test (MAT), which is recommended by the OIE, was performed (http://www.oie.int/doc/ged/D12009.PDF). Antigens representing serovars Canicola, Grippotyphosa, Pomona, Icterohaemorrhagiae, Tarassovi, Ballum, Bratislava and Hardjo-bovis were used. Initially, two-fold serum dilutions were used from 1:50 to 1:200. Positive samples were diluted until an end point was reached. The final tier of a sample was the last dilution of sera in which agglutination of at least 50% of antigen was observed.

IFAT for Neospora caninum (N. caninum)

The presence of antibodies to N. caninum was performed by Indirect Fluorescent Antibody Test (IFAT) as described by [10].

**Results**

**Routine EHDV diagnosis (field samples)**

A total of 152 (out of 321) field samples from cattle showing clinical signs were found positive for EHDV by RT-qPCR. One hundred and eighty three were sampled between October and November 2015, of which more than half (105 samples) were positive. Thereafter, the number of field samples and percentage of positive results decreased gradually until March 2016. From April 2016 onwards, no positive field samples were detected [4] (Table 1).

### Table 1: Total and percent positive clinical samples by PCR and virus isolation, July 2015 to April 2016.

| Date       | Adult samples* | VI in ECE* | Aborted fetuses* |
|------------|----------------|------------|------------------|
| Jul-2015   | 0/32-0%        | NT         | NT               |
| Aug-2015   | 0/7-0%         | NT         | NT               |
| Sep-2015   | 5/7-71%        | 2/5-40%    | 0/1-0%           |
| Oct-2015   | 57/95-60%      | 7/56-12.5% | 2/9-22.2%        |
| Nov-2015   | 58/88-65.9%    | 3/57-5.3%  | 3/5-60%          |
| Dec-2015   | 17/46-36.9%    | 0/17-0%    | 6/11-54.5%       |
| Jan-2016   | 7/17-41.1%     | 0/2-0%     | 0/4-0%           |
| Feb-2016   | 2/15-13.3%     | NT         | NT               |
| Mar-2016   | 7/49-14.3%     | 0/2-0%     | NT               |
| Apr-2016   | 0/23-0%        | NT         | NT               |

*Presented as: positive / total samples-percent

**Table 2: EHDV RT-qPCR results and virus isolation from whole blood samples from six monitored naturally infected calves**

| Date of sampling | Ct qPCR values | ECE VI* | CC VI* |
|------------------|----------------|---------|--------|
| 12-Oct-2015      | 21.7-27.3      | 0/5     | 0/5    |
| 17-Nov-2015      | 23.4-27.4      | 0/6     | 0/6    |
| 8-Dec-2015       | 23.4-30.8      | 0/6     | 0/6    |
| 11-Feb-2016      | 26.1-34.9      | NT      | NT     |
| 31-Mar-2016      | NA             | NT      | NT     |

**Table 3: Monitoring of EHDV-6 RNAmia and antibodies in naturally infected calves**

The EHDV-6 RNA levels in whole blood samples from monitored calves were quantified by RT-qPCR. The highest quantity of EHDV-6 RNA (21.7 - 27.4 Ct) was present in all whole blood samples from all calves during the first two months from the presumed date of infection, after which a gradual decline till undetectable by five months from the presumable date of infection (Table 2).

**Gross pathology examination**

Bovine fetuses with or without placenta were examined post mortem. Tissue samples of lung, heart, spleen, kidney, brain and placenta were fixed in normal buffered 10% formaldehyde for future histopathological examination (The results will be published in a separate report in future).
Seroconversion was detected in all six calves on the 12th of October 2015, but no clinical signs of disease were apparent.

The monitored calves did not show any clinical signs of the disease, as opposed to several dairy cows from the same farm.

**Gross pathological changes in aborted fetuses and placentas**

Most of the fetuses received for necropsy were at different stages of autolysis, with additional microbial contamination. Only in one fetus, hemorrhages in the heart (right atrium) were seen. In placental lesions of autolysis, with additional microbial contamination. Only in one placenta sample of the monitored naturally infected calves, attempts to obtain virus isolates both using ECE and two different cell culture lines (Vero and BHK-21) failed (Table 2). EHDV-6 isolation in ECE was successful only in one of five RT-qPCR positive whole blood samples from aborted cows with clinical signs within the same farm (data not shown), during the same time period.

**Virus isolation**

Infectious EHDV was successfully isolated in 12 out of 139 EHDV-RF-qPCR positive whole blood samples taken from clinically diseased animals. Two isolates were obtained from samples received in September 2015, seven in October 2015 and three in November 2015. No virus was isolated after November 2015 (Table 1).

**Laboratory findings from aborted fetuses**

From September through January 2016, 30 aborted bovine fetuses were tested for potential abortogenic pathogens including EHDV, Simbu group viruses, BVD, Leptospirosis, Brucellosis and N. caninum. Antibodies to Leptospira borgpetersenii serovars Harjio were detected in the serum of one of the aborting dams (Table 3), while all were negative for Brucella spp. Eleven aborted fetuses were EHDV positive by RT-qPCR, four - for Simbu group viruses by PCR (SHUV and AKAV), two -for BVD (Ag ELISA), eleven fetuses had antibodies against N. Caninum by IFAT and Trueperella pyogenes was isolated from one placenta.

Six of the thirty aborted bovine fetuses were negative (Table 3) for all tested abortion-related pathogens (routine diagnosis).

EHDV RNA was found in 7 out of 16 (43.75%) brain and 6 out of 13 (46.15%) placenta samples. All samples from fetal internal organs were negative for EHDV. The first EHDV positive sample from aborted fetus was found at the beginning of October, and the last one in mid-December. The majority of infected aborted cattle fetuses were collected during November and December of 2015 (Table 1 and 3). Generally, eleven of thirty aborted fetuses were PCR-positive for EHDV. EHDV as the sole abortive agent was detected in seven fetuses, while in the remaining four EHDV positive samples additional abortogenic pathogens were identified (Table 3).

**Other potential abortigenic factors**

All cases sampled in this study were from intensively managed zero-grazing dairy cattle. The balanced, whole-feed rations were monitored for the presence of nitrates and mycotoxins by the feed producers and were distributed to several other holdings with no apparent detrimental effects.

No aberrant use of pesticides was reported on these farms and no indications of the presence of toxic substances were noted.

**Discussion**

The naturally EHDV-6 infected sentinel calves observed in this study, did not exhibit clinical signs, suggesting probable subclinical infection. This is consistent with previous findings where calves experimentally infected with Turkish and Moroccan isolates of EHDV-6 also remained healthy and no clinical signs were observed [11]. EHDV RNAemia was seen in the blood in calves at least four months from the presumable date of natural infection based on seroconversion and qPCR. Despite high RNA levels detected in the blood samples of the monitored naturally infected calves, attempts to obtain virus isolates both using ECE and two different cell culture lines (Vero and BHK-21) failed (Table 2). EHDV-6 isolation in ECE was successful only in one of five RT-qPCR positive whole blood samples from affected cows with clinical signs within the same farm (data not shown), during the same time period.

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**Table 3:** Laboratory findings from aborted fetuses, which were tested for EHDV genome presence by specific RT-qPCR.

| Date       | Lab Num   | Lab diagnosis of aborted fetuses |
|------------|-----------|---------------------------------|
| day/m/y    |           | Viral | Bact | Parasite | Pathol |
| 20/09/15   | 188/15⁴   | -     | -    | -        | -      |
| 08/10/15   | 192/15⁵   | EHDV | -    | -        | -      |
| 08/10/15   | 193/15⁰   | SHUV | -    | -        | N. caninum Mild placentitis |
| 13/10/15   | 195/15¹   | -     | -    | -        | -      |
| 14/10/15   | 198/15²   | -     | -    | -        | -      |
| 21/10/15   | 201/15⁶   | EHDV | mix.cul | - | Autoletic |
| 25/10/15   | 203/15⁷   | -     | mix.cul | - | -      |
| 26/10/15   | 204/15⁸   | -     | -    | N. caninum | Hemorrhage in the right atrium |
| 27/10/15   | 205/15⁹   | -     | -    | -        | Multifocal hepatitis |
| 27/10/15   | 206/15¹⁰  | -     | -    | -        | -      |
| 08/11/15   | 208/15¹¹  | EHDV | -    | -        | N. caninum Myocarditis |
| 19/11/15   | 213/15¹²  | -     | -    | -        | -      |
| 19/11/15   | 214/15¹³  | EHDV/ShUV/BDV L. hardjo ** | - | Large cysts in kidney |
| 23/11/15   | 221/15¹⁴  | EHDV | -    | -        | -      |
| 25/11/15   | 224/15¹⁵  | SHUV | -    | -        | Autoletic |
| 01/12/15   | 230/15¹⁶  | EHDV | -    | -        | -      |
| 03/12/15   | 231/15¹⁷  | EHDV | -    | -        | N. caninum Myocarditis |
| 07/12/15   | 233/15¹⁸  | -    | T. pyogenes | - | -      |
| 09/12/15   | 237/15¹⁹  | EHDV | -    | -        | -      |
| 13/12/15   | 244/15²⁰  | EHDV/ShUV/BDV/ AKAV/ SHUV | - | Epi/endo-carditis, periportal hepatitis |
| 15/12/15   | 248/15²¹  | EHDV | -    | -        | -      |
| 16/12/15   | 249/15²²  | -    | N. caninum | - | -      |
| 17/12/15   | 250/15²³  | -    | N. caninum | Epi/endo-carditis, periportal hepatitis |
| 21/12/15   | 253/15²⁴  | SHUV | mix.cul | - | -      |
| 21/12/15   | 255/15²⁵  | EHDV | mix.cul | - | -      |
| 31/12/15   | 259/15²⁶  | -    | mix.cul | N. caninum | - |
| 04/01/16   | 101/1⁷²⁶  | -    | mix.cul | - | -      |
| 05/01/16   | 102/1⁷²⁶  | BVD | -    | -        | Periportal hepatitis |
| 26/01/16   | 117/1⁷²⁶  | -    | mix.cul | N. caninum | Autoletic |
| 31/01/16   | 120/1⁷²⁶  | -    | -    | N. caninum | - |

[Table 3] Laboratory findings from aborted fetuses, which were tested for EHDV genome presence by specific RT-qPCR.

* - Brain sample
⁴ - Placenta sample
⁵ - Both in placenta and brain samples viral genome was identified
⁶ - Mixed sample from different organs
⁷ - Antibodies were found in serum of the aborted cow
⁸ - Trueperella pyogenes
mix.cul - mixed culture
Multifocal mononuclear infiltrates were seen in two brain samples, which were positive only by EHDV RT-qPCR leading to a diagnosis of encephalitis (data not shown in this report).

No gross changes were seen in any tested placentas. Additionally, in a few samples, antibodies against N. caninum were found (Table 3).

Bluetongue virus (BTV) is closely related to EHDV, is known to be capable to cross the placental barrier, as seen with the attenuated vaccine strains belonging to serotypes 2, 9, 10, 11, 13 and 17 [12,13], as well as with wild type BTV-8 and BTV-1 [14]. Congenital abnormalities (e.g., hydranencephaly, cavitary encephalopathy, cerebral hyperaemia) have been observed in the field as well as in experimental studies following transplacental BTV-8 infection of fetuses [15-18]. No placental lesions were observed in experimental infection of a pregnant goat with BTV-8 [15], as well as EHDV positive placentas, investigated during this outbreak.

Previously, only the EHDV-2 strain (Ibaraki virus) was considered to cause abortion, in addition to severe clinical disease in cattle [19,20]. In this study, the absence of commonly diagnosed abortifacient pathogens of cattle in Israel implicates the involvement of EHDV-6 in abortions seen in some of the cattle population from September 2015 until January 2016. EHDV was the only pathogen identified in 7 out of 30 aborted fetuses. Consequently, EHDV-6 may be considered implicated in the abortions in other cows during this period, where no other agents were detected.

It should, however, be noted that the cases of abortion occurred several weeks after the presumed onset of the EHDV-6 outbreak, emphasizing the delay from the appearance of viremia to fetal loss. The presence of the virus in the brains of aborted fetuses suggests that fetal infection took place several weeks prior to fetal death in utero. The high percentage of EHDV-positive placentas samples (7 out of 13 for EHDV-6 -46.15%, Table 3) indicates that this could be a valuable diagnostic tool to confirm viral infection in cases of bovine abortion as was found previously with Bovine Herpesvirus-1 and 4 infection [21,22] and SBV [23].

Considering all tested abortogenic pathogens, our finding of EHDV-6 viral genome in aborted cattle fetuses suggest that the virus was significant in inducing abortion in cattle during that period.

**Conclusion**

Based on the data from this study, the EHDV-6 outbreak in Israel lasted between late September and November 2015. Our findings indicate that the duration of RNAemia in naturally infected calves lasted approximately four months. However, the duration of viremia is still unclear and, possibly, significantly shorter.

**Conflict of Interest**

The author(s) declared no potential conflict of interest with respect to the research, authorship, and/or publication of this article.

**Acknowledgement**

This work was partly supported by the Ministry of Agriculture Chief Scientist grant # 33-03-0004.

We are very grateful to the teams of the Field Government Veterinary Services for sampling the sentinel cattle, to all the veterinary practitioners in the field for collaboration, to Mrs. Veronika Peper, to Dr. Anita Koftunenko and to Mrs. Anna Gorohov for excellent technical assistance, to Dr. Avi Eldar, to Dr. Oran Erster, to Dr. Jacob Brenner and to Dr. Alexander Panshin for very useful scientific advises.

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