

Experimental infection of calves with seven serotypes of Epizootic Hemorrhagic Disease virus: production and characterization of reference sera

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Keywords

Epizootic hemorrhagic disease virus,
Experimental infection,
Reference sera,
Serological diagnosis.

Summary

The aim of this study was to produce reference sera against the seven serotypes of Epizootic hemorrhagic disease virus (EHDV-1, EHDV-2, EHDV-4, EHDV-5, EHDV-6, EHDV-7, and EHDV-8). In a high containment unit, seven Prim' Holstein calves were inoculated at day 0 (D0) with the selected strains (1 EHDV serotype per calf). Blood samples (EDTA and whole blood) were periodically taken from D0 until the end of the experiment (D31). Sera were tested with two commercially available EHDV competitive ELISAs (c-ELISA). Viral genome was detected from EDTA blood samples using in-house real-time RT-PCR. Sera taken on D31 post infection (pi) were tested and characterized by serum neutralization test (SNT) and virus neutralization test (VNT) (for calibration of reference sera). Viral RNA was first detected at D2 pi in five calves. All infected animals were RT-PCR positive at D7 pi. Seroconversion was observed between D10 and D23 pi depending on the EHDV serotype. SNT and VNT have allowed to determine the neutralizing antibody titers of each serum and the potential cross-reactions between serotypes. The two c-ELISA used in this study showed similar results. The calibrated sera are now available for the serological identification of an EHDV isolated on tissue culture or to be used as positive control in seroneutralization assay.

Infezione sperimentale di vitelli con sette sierotipi del virus della malattia emorragica epizootica: produzione e caratterizzazione di sieri di riferimento

Parole chiave

Virus della malattia emorragica epizootica,
Infezione sperimentale,
Sieri di riferimento,
Diagnosi sierologica.

Riassunto

Scopo di questo studio è stato produrre sieri di riferimento contro i sette sierotipi del virus della malattia emorragica epizootica (EHDV-1, EHDV-2, EHDV-4, EHDV-5, EHDV-6, EHDV-7 e EHDV-8). In una struttura BSL-3 sono stati inoculati sette vitelli Prim' Holstein con i ceppi di EHDV selezionati (1 sierotipo per vitello). Campioni di sangue (EDTA e sangue intero) sono stati prelevati periodicamente il giorno dell'inoculo (D0) fino alla fine dell'esperimento (D31). I sieri sono stati testati con due kit ELISA (c-ELISA). Il genoma virale è stato rilevato da campioni di sangue EDTA mediante una *in-house real-time RT-PCR*. Per calibrare i sieri di riferimento, i sieri prelevati al D31 sono stati testati e caratterizzati sia con il test di sieroneutralizzazione (SNT) che con il test di virusneutralizzazione (VNT). Due giorni dopo l'inoculo 5 animali sono risultati positivi alla RT-PCR specifica per EHDV. A sette giorni tutti gli animali erano positivi. Tutti gli animali hanno sierconvertito a seconda del sierotipo tra D10 e D23, in base al sierotipo EHDV, tra D10 e D23. I saggi di siero- e virusneutralizzazione hanno permesso di determinare i titoli anticorpali neutralizzanti di ciascun siero e le potenziali reazioni crociate tra sierotipi. I due kit c-ELISA utilizzati in questo studio hanno mostrato risultati simili. Gli antisieri così prodotti e calibrati sono disponibili e possono essere utilizzati per identificare ceppi di EHDV isolati in tessuto colture o come controlli positivi nel test di sieroneutralizzazione.

Introduction

Epizootic hemorrhagic disease (EHDV) is an arthropod-borne disease of wild and domestic ruminants caused by viruses (EHDV) belonging to the species EHDV within the genus *Orbivirus* of the *Reoviridae* family (Mertens *et al.* 2005). Seven serotypes are officially recognized (EHDV-1, EHDV-2, EHDV-4, EHDV-5, EHDV-6, EHDV-7, and EHDV-8) and more recently at least 2 putative new serotypes have been reported (Maan *et al.* 2010, Shirafuji *et al.* 2017, Anthony *et al.* 2009, Savini *et al.* 2011).

As the clinical pattern is very close to Bluetongue infection, laboratory investigations are essential for the diagnosis of the EHDV infection. In most infected areas, Bluetongue and EHD viruses co-circulate (Dommergues *et al.* 2019, Merrill *et al.* 2019, Toye *et al.* 2013) and mixed infections are reported (Sailleau *et al.* 2012, Viarouge *et al.* 2014). This report highlights the need for efficient, specific and sensitive diagnostic tools to identify the causal agent.

The molecular tools for EHDV nucleic acid identification are widely used for the diagnosis. In the last ten years, real-time RT-PCR assays (rtRT-PCR) have been developed for the detection of all seven serotypes (Clavijo *et al.* 2010, Maan *et al.* 2017, Viarouge *et al.* 2015, Wilson *et al.* 2009) and the typing of the virus (Maan *et al.* 2017, Viarouge *et al.* 2015). Serological tools including ELISA have been described for the EHDV antibody detection (Afshar *et al.* 1992, Thevasagayam *et al.* 1995) and two commercial kits were launched in 2010 and 2017. These serological tools allow a serogroup EHDV antibody detection without identification of EHDV serotype antibodies. These are competitive or blocking ELISA using an HRP-labelled monoclonal against the viral protein 7 (VP7). The serum neutralization test (SNT) is usually performed to identify exposure to specific EHDV serotypes (OIE 2014).

Even if molecular diagnosis is widely used, virus isolation must be performed for a reliable diagnosis and, particularly, in the case of an emergence. Moreover, this viral isolation is needed to facilitate further pathogen characterization and pathogenesis studies. Following the viral isolation, reference sera are used in virus neutralization test (VNT) for the serotype determination (OIE 2014, Pearson *et al.* 1992). The aim of this study was to produce and characterize reference sera against EHDV serotypes.

Materials and methods

Experimental infection of calves

Seven Prim'Holstein calves (2 to 3 months-old) were housed in a high containment unit (Centre

de Recherche BioMédicale, Maisons-Alfort, France - CRBM) throughout the experiment. Calves were confirmed negative for EHDV using a commercial kit for EHDV antibodies detection (ThermoFisher, Lissieu, France) and a pan EHDV rtRT-PCR assay previously developed in our lab (Viarouge *et al.* 2015). The serological and virological status regarding BTV was also checked. The animals were BTV-free.

At Day 0 (D0), all calves were inoculated intramuscularly at multiple sites in the shoulder region with 5-6 ml of inoculums (EHDV culture cell supernatants). EDTA-treated whole-blood and serum samples were collected at regular intervals until the end of the experiments up to D31 post-infection (pi). Hundreds of ml of blood were collected from each animal before euthanasia to produce a reference serum for each serotype. The EHDV-7 infected calf was euthanized at 17 days because it showed severe clinical signs.

Table I. Viral strains used to calves inoculation and to VNT.

Strain	Origin	Titre TCID ₅₀ /ml	Used for
EHDV-1 USA1955/01	Ref strain	2.5 10 ⁶	Inoculation of calf 7706/VNT
EHDV-2 CAN1962/01	Ref strain	1.10 ⁷	Inoculation of calf 7713/VNT
EHDV-4 NIG1968/01	Ref strain	5.6 10 ⁵	Inoculation of calf 7716/VNT
EHDV-5 AUS1979/06	Ref strain	3.2 0 ⁵	Inoculation of calf 7724/VNT
EHDV-6 AUS1981/07	Ref strain	2.3 10 ⁶	Inoculation of calf 7730/VNT
EHDV-7 ISR2006/01	Israël 2006	5.6 10 ⁵	Inoculation of calf 7739/VNT
EHDV-8 AUS1982/06	Ref Strain	1.10 ⁴	Inoculation of calf 7748/VNT
EHDV-1 Guy	French Guyana (2011)	3.2 10 ⁴	VNT
EHDV-1 Reu	Réunion Island (2011)	1.8 10 ⁴	VNT
EHDV-2 AUS1979/05	Australia (1979)	1.8 10 ⁶	VNT
EHDV-2 JAP/01	Japon	1.8 10 ⁷	VNT
EHDV-2 Guy	French Guyana (2014)	1.8 10 ⁴	VNT
EHDV-2 Guad	Guadeloupe Island (2011)	3.2 10 ⁶	VNT
EHDV-6 Mor	Morocco (2007)	3.2 10 ⁶	VNT
EHDV-6 Reu	Réunion Island (2009)	5.6 10 ³	VNT
EHDV-6 Guy	French Guyana (2016)	5.6 10 ³	VNT
EHDV-7 AUS1981/05	Ref strain	2.5 10 ⁴	VNT
EHDV-7 Reu	Réunion Island (2014)	1.8 10 ⁶	VNT

Ethic

All experimental protocols were reviewed by a state ethics commission and have been approved by the competent authority (reference: 2017051515187349). All adequate measures were taken to minimize pain or discomfort.

Viruses

The seven EHDV strains used for the inoculation of calves (Table I) have been titrated and tested with the seven real-time serotyping RT-PCR assays (Viarouge *et al.* 2015) in order to control their purity (absence of other serotypes). All strains were passaged on KC cells (*Culicoides sonorensis* cell line) (Wechsler *et al.* 1989) before the inoculation of calves. As EHDV-8 failed to grow on KC cells, a BSR passage was performed to produce the EHDV-8 inoculum.

Real time PCR

Total RNA was extracted from 100 μ L of EDTA-blood using the Kingfisher 96 robot and the MagVet Universal isolation kit (ThermoFisher, Cambridge, UK) according to manufacturer's instructions. Finally, the RNAs were eluted with 80 μ L of ultrapure water and 5 μ L were used, after denaturation at 95 °C for 3 minutes, in pan-EHDV rtRT-PCR assay (Viarouge *et al.* 2015).

Serological analyses

ELISA

The detection of EHDV VP7 antibodies was performed using a competitive ELISA (ThermoFisher, Lissieu, France). Two negative controls (Cneg) and two positive controls (Cpos) were analysed in duplicate on each plate. The results were expressed as inhibition percentage

$$(\%inh) = [\text{optical density (OD) of the Cneg} - \text{OD sample} / \text{OD Cneg}] * 100.$$

The assay was performed according to manufacturer's instructions (%inh > 60%: positive; %inh < 55%: negative and %inh between 55 and 60%: doubtful). This kit is no longer available.

The serum samples were also tested with another cELISA (ID Screen® EHDV Competition, IDvet, Grabels, France) following the manufacturer's instructions. Two negative controls (Cneg) and two positive controls (Cpos) were analysed in duplicate on each plate. The results were expressed as

$$\text{Sample/Negative Control (S/N)} = (\text{optical density (OD) of the sample} / \text{OD Cneg}) * 100.$$

(S/N < 30%: positive; S/P > 40%: negative and S/N between 30 and 40%: doubtful).

Serum neutralization test (SNT)

Sera sampled at the last day of the study (D17 pi for EHDV-7 infected calf and D31 pi for the others) were titrated for the presence of serotype specific EHDV antibodies by SNT. Each serum was tested against all EHDV serotypes.

Briefly, two-fold dilutions of sera (in duplicate) were done in DMEM Glutamax Medium (GIBCO, Grand Island, New York), starting at $\frac{1}{2}$ (range of dilution: 2-256). Fifty microlitres of DMEM, containing 100 TCID₅₀ of each EHDV serotype, were incubated in microtitre plates, with 50 μ L of the diluted sera for 1 hour at 37 °C. Then, 20,000 BSR cells were added in each well, with 100 μ L of DMEM supplemented with 10% fetal calf serum and 2% sodium pyruvate. Microtitre plates were then incubated for 6-7 days at 37°C. The neutralizing titre of each serum was defined as the highest dilution allowing 50% neutralisation of the 100 TCID₅₀. The titers thus defined has been used to determine the four neutralizing unities (NU) required for the VNT. The serum titre gives the value of the one neutralizing unit. For example, if the titer is 128 the dilution to do to obtain 4 NU will be 1/32.

Virus neutralization test (VNT)

Each reference serum was evaluated against its homologous EHDV serotype and against those where cross-reactions were observed in SNT.

The reference sera were diluted in MEM to obtain four NU. VNT was carried out in microtitre plates. Briefly, four NU of reference sera (50 μ L) were added to a 10-fold dilution series of EHDV (50 μ L). The serum-virus mixtures were incubated for 1 h at 37 °C. As performed for SNT, BSR cells (20,000 cells in 100 μ L/well) were then added and the plates were incubated for 6 days at 37 °C (Sailleau *et al.* 2000). A two-way reduction of at least 2 logs is considered to classify a virus as belonging to the serotype of the antibody which neutralized it.

Results

Clinical observations

The calves infected with EHDV serotypes 1, 2, 4, 5, 6 and 8 did not show apparent clinical signs until D31. Only the EHDV-7 infected calf was euthanized at 17 days post inoculation because it showed severe clinical signs (apathy, diarrhea, prostration, unable to stand, anorexia).

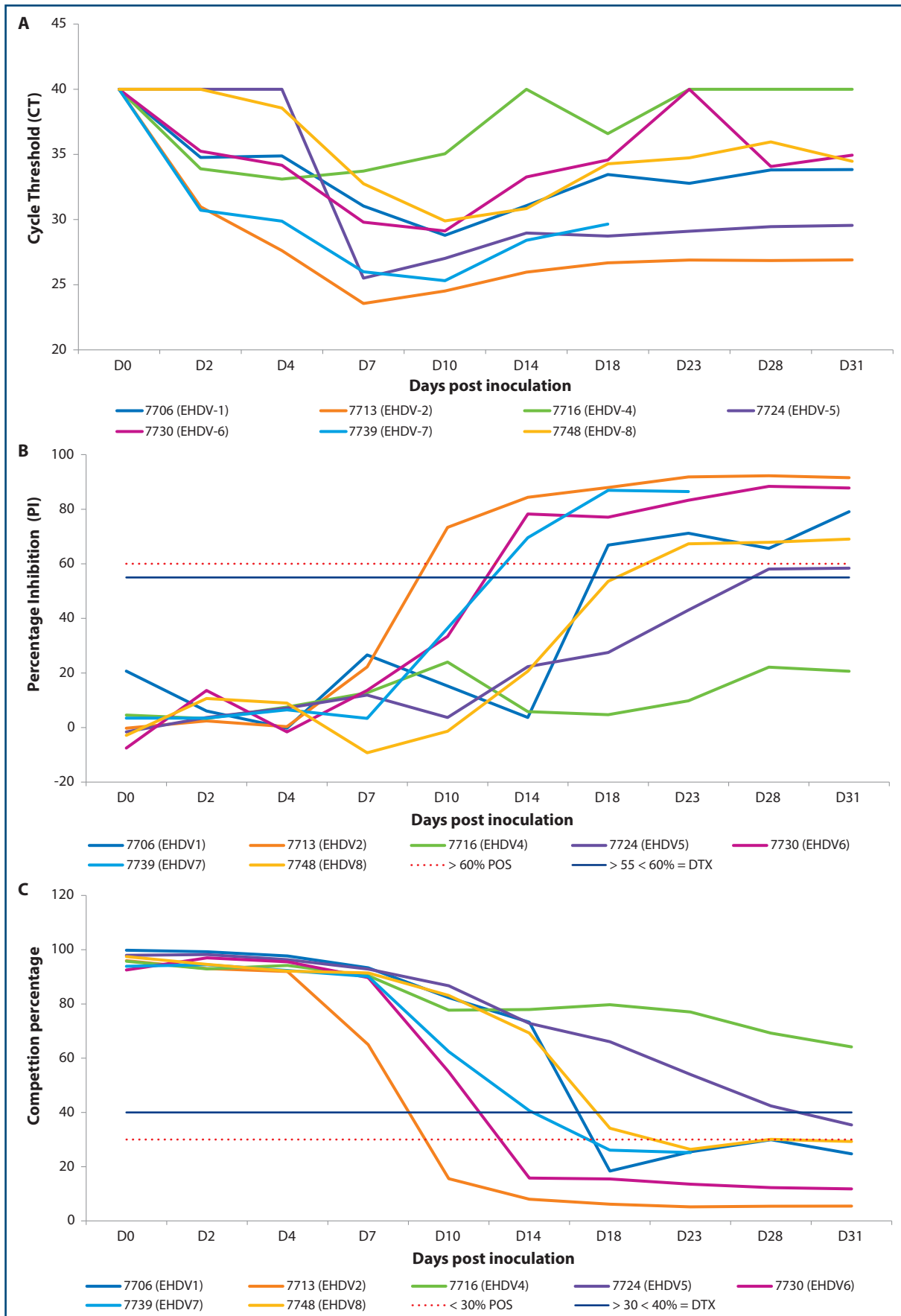


Figure 1. Follow-up of the biological parameters in calves after experimental infection with different Epizootic hemorrhagic disease virus (EHDV) serotypes. **A.** Viral genome (Real time RT-PCR assay); anti EHDV VP7 antibodies. **B.** LISIVET EHDV Blocking, Thermofisher, France. **C.** ID Screen® EHDV Competition - Idvet France.

Follow-up of the serological and virological parameters

rtRT-PCR

Before infection (at D0), all animals were EHDV rtRT-PCR negative. Viral RNA was first detected at D2 pi in calves inoculated with EHDV-1, EHDV-2, EHDV-4, EHDV-6, and EHDV-7. All animals were rtRT-PCR positive at D7 pi. Five out of 7 animals remained EHDV rtRT-PCR positive until the end of the experiment. The EHDV-4 infected calf became rtRT-PCR negative from D14 pi. The EHDV-7 infected calf remained positive until D17 pi (date of euthanasia) (Figure 1A).

ELISA results

There was a complete agreement between the results obtained with the two ELISAs used (Figures 1B and 1C). Animals inoculated with EHDV-1, EHDV-2, EHDV-6, EHDV-7 and EHDV-8 seroconverted between D10 and D23 pi. The EHDV-4 infected calf failed to seroconvert and low level of anti-VP7 antibodies were detected between D28 and D31 pi in the animal infected with EHDV-5.

The reference sera from the calves inoculated with serotype 4 or 5 were found negative and doubtful, respectively (close to the positivity threshold) when tested with both ELISA kits. The reference sera from EHDV-1, EHDV-2, EHDV-6, EHDV-7, and EHDV-8 were ELISA-positive.

Table II. Serum neutralization test results obtained with Epizootic hemorrhagic disease virus (EHDV) antisera. The Titres are expressed as the reciprocal value of the endpoint serum dilution. Shaded cells: titre of sera with their homologous serotype. In bold: titres of sera with heterologous serotypes (cross-reaction). In bracket: the dilution used for each reference serum to obtain 4 Neutralizing Unit.

Virus	Antisera						
	7706 (EHDV-1)	7713 (EHDV-2)	7716 (EHDV-4)	7724 (EHDV-5)	7730 (EHDV-6)	7739 (EHDV-7)	7748 (EHDV-8)
EHDV-1	128 (1/32)	8	< 4	< 4	< 4	4	< 4
EHDV-2	8	64 (1/16)	< 4	< 4	4	32	4
EHDV-4	< 4	< 4	128 (1/32)	8	< 4	< 4	< 4
EHDV-5	< 4	< 4	16	384 (1/96)	< 4	< 4	< 4
EHDV-6	< 4	< 4	< 4	< 4	96 (1/24)	4	16
EHDV-7	< 4	8	< 4	< 4	< 4	128 (1/32)	< 4
EHDV-8	< 4	< 4	< 4	< 4	< 4	< 4	32 (1/8)

Table III. Virus neutralization test results obtained with different Epizootic hemorrhagic disease virus (EHDV) strains against homologous and heterologous antisera. Values represent the log reduction in cytopathic effect (neutralization). A two-way reduction of at least 2 logs is considered to classify a virus as belonging to the serotype of the antibody which neutralized it.

Antisera	Virus							
	EHDV-1 USA1955/01	EHDV-1 Guy	EHDV-1 Reu	EHDV-2 Guy	EHDV-2 Guad	EHDV-7 AUS1981/057	EHDV-7 ISR2006/01	EHDV-7 Reu
7706 (EHDV-1)	2.4	2	2					
7713 (EHDV-2)	2	1.46	4.4	2	2.71	2.7	1.5	1.25
7716 (EHDV-4)	2.4	1.4						
7724 (EHDV-5)	2.7	1						
7730 (EHDV-6)	3.4	1.9	2					1.7
7739 (EHDV-7)	> 2.7	3.5	2					1
7748 (EHDV-8)	> 3	2.39	1.51	2				

SNT and VNT

SNT and VNT were performed only with the reference sera. The seven sera were titrated against all serotypes of EHDV in order to detect potential cross-reactivity (Table II). The titres obtained in the SNT performed with homologous serotypes ranged between 32 and 384. Weak cross-reactions (titre = 8) were observed between reference sera of EHDV-1 and EHDV-2, EHDV-2 and EHDV-1 and EHDV-7, EHDV-5 and EHDV-4. Relative strong cross reactions (titers = 16 or 32) were obtained between sera of EHDV-4 and EHDV-5, EHDV-7 and EHDV-2, and EHDV-8 and EHDV-6.

In order to confirm and complete the serological data generated by SNT (titres/cross-reaction), VNT was performed by using several EHDV strains (Table III).

Each reference serum neutralized all homologous strains except EHDV-2 antiserum, which neutralized only 4 of 5 EHDV-2 strains tested. EHDV-2 JAP was only partially neutralized (reduction of 1.46). Similarly to SNT results, cross-reactions EHDV-2 reference serum neutralized EHDV-7 AUS1981/05 but not the other EHDV-7 strains, while EHDV-8 reference serum cross-neutralized EHDV-6 AUS1981/07 and EHDV-6 Mor.

Discussion

No case of EHDV infection has been so far reported in Europe but the risk of introduction is very high. In the last 15 years, EHDV-1, EHDV-6 and EHDV-7 have been detected in the south or east of the Mediterranean basin (the Maghreb, Egypt, Israel, Turkey, and Jordan) (Ahmed *et al.* 2019, Ben Dhaou *et al.* 2016, Golender *et al.* 2017, Temizel *et al.* 2009).

The availability of reliable and standardized reagents for laboratory diagnosis is crucial in the event of the emergence of this disease. The main purpose of this experimental infection was to produce reference sera to be used in serological assays, either to identify by VNT the EHDV isolated on tissue cultures (unknown serotype against known sera), or as positive controls in SNT (unknown sera against known serotypes).

Of the seven animals infected with different EHDV serotypes, six remained healthy throughout the entire experimental period and no clinical signs of EHD were observed. The calf inoculated with the EHDV-7 ISR2006/01 strain was euthanized at D17 pi. The clinical signs observed (apathy, diarrhea, prostration, unable to stand and anorexia) were different from those described during the 2006 epizootic in Israel (Yadin *et al.* 2008). In a similar experimental infection of Holstein cattle using the same strain, no clinical signs were reported (Eschbaumer *et al.* 2012). As a post mortem examination of the experimentally infected animals

could not be carried out, it is not possible to assert that the symptoms observed in the infected calf were partially or totally due to EHDV-7 infection.

Similarly to what observed by other authors, also in this study most of the infected animals become EHDV PCR positive from D2 pi (Batten *et al.* 2011, Breard *et al.* 2013, Eschbaumer *et al.* 2012). Except for EHDV-4, all animals remained PCR positive until the end of the experiment. Depending on the serotype inoculated, seroconversion (ELISA) was observed between D10 (EHDV-2) and D23 pi (EHDV-8). The serum from EHDV-5 inoculated calf gave doubtful results from D28 pi. Surprisingly, the EHDV-4 infected calf remained negative in ELISA during the entire experimental period while the SNT showed a strong titer (128) at D31 pi. This result could be due to the fact that the anti-VP7 antibodies produced against EHDV-4 have a low affinity for the epitope on which the competition with the anti-VP7 monoclonal (used in both ELISA kits) is performed. The same hypothesis can be formulated for the EHDV-5 serum, which gave doubtful results with both ELISAs. Altogether, the results obtained with the two commercial c-ELISA kits used in this trial were perfectly similar (Figures 1B and 1C). More importantly, the four serotypes (1, 2, 6 and 7) mainly circulating in the world were well detected by both ELISAs.

The neutralizing antibody titres obtained for the seven reference sera ranged from 32 to 384 (Table II). The close antigenic relationship between some serotypes has already been reported in previous studies (Anthony *et al.* 2009, Maan *et al.* 2010). Both in SNT and VNT, strong cross-reactions were found between EHDV-2 and EHDV-7 and EHDV-6 and EHDV-8. However, these cross-reactions were not observed for all isolates: EHDV-2 reference serum neutralized EHDV-7 AUS1981/05 (log reduction in CPE 2.7) but not other EHDV-7 strains (EHDV-7 Isr and EHDV-7 Reu) (log reduction in CPE < 2).

Anthony and colleagues (Anthony *et al.* 2009) have suggested that EHDV-2 and EHDV-7, and EHDV-6 and EHDV-8 have diverged (or are still diverging) from two different common ancestors. Some isolates which were weakly neutralized by their homologous antiserum (e.g. EHDV-6 Reu and EHDV-2 JAP/01) (Table III) probably have a lower antigenic relationship with the isolate used for the production of serum in calf.

Despite the cross-reactions described, the use of the seven calibrated antisera in VNT made the serotype identification of an EHDV infected tissue culture possible.

To date conventional or real-time RT-PCRs are commonly used for fast and reliable identification of EHDV (Maan *et al.* 2010, Maan *et al.* 2017, Viarouge

et al. 2015). However, the genetic variations that may appear over time in the EHDV genome (in region where specific primers/probes for typing assays are designed) can cause the failure of the RT-PCR. Therefore, virus isolation in tissue culture and VNT should be kept as the gold standard for detecting and serotyping EHDV isolates. Specific and reliable antisera are therefore essential for this serological test.

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