Experimental infection of cattle, sheep, and goats with the newly emerged epizootic hemorrhagic disease virus serotype 8

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Summary
Epizootic hemorrhagic disease virus serotype 8 (EHDV-8) emerged in Europe for the first time in late 2022. In this study, we investigated the kinetics of EHDV-8 infection in cattle, sheep, and goats. Following experimental infection with EHDV-8, three out of five calves displayed fever, and one calf exhibited ulcerative and crusty lesions of the muzzle. RNAemia peaked at day 7 post infection in all calves and remained relatively stable till the end of the study, at 78 days post infection. Infectious virus was isolated up to 21 days post infection in one calf. As far as small ruminants are concerned, one sheep experienced fever and two out of five had consistent RNAemia that lasted until the end of the study. Remarkably, infectious virus was evidenced at day 7 post infection in one sheep. In goats, no RNA was observed in blood samples. All infected animals seroconverted, and a neutralizing immune response was observed in all species, with calves exhibiting a more robust response than sheep and goats. Our study provides insights into the kinetics of EHDV-8 infection and the host immune responses. We also highlight that sheep may also play a role in EHDV-8 epidemiology. Altogether, the data gathered in this study could have important implications for disease control and prevention strategies, providing crucial information to policy makers to mitigate the impact of this viral disease on livestock.

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Introduction

Epizootic hemorrhagic disease (EHD) is a World Organization of Animal Health (WOAH)-notifiable vector-borne viral disease of certain species of wild and domestic ruminants. The virus is transmitted from infected to naive animals by *Culicoides* female biting midges (Savini *et al*., 2011).

EHD is caused by EHD virus (EHDV), an *Orbivirus* (family *Sedoreoviridae*) closely related to bluetongue virus (BTV), the causative agent of bluetongue (BT) disease of ruminants. EHDV genome consists of 10 segments (S1-S10) of dsRNA encoding for 7 structural (VP1 to VP7) and 5 non-structural proteins (Jiménez-Cabello *et al*., 2023). Currently, seven provisional serotypes (1, 2, and 4-8) have been described based on neutralization assays and phylogenetic studies (Anthony *et al*., 2009; Maclachlan *et al*., 2015; Maclachlan *et al*., 2019).

While outbreaks in wild and captive populations of North American white-tailed deer (*Odocoileus virginianus*) are characterized by high morbidity and mortality (Fischer *et al*., 1995; Gaydos *et al*., 2004; Savini *et al*., 2011), infections of cattle (*Bos taurus*), with the notable exception of the Ibaraki strain of EHDV-2, have been considered asymptomatic or pauci-symptomatic for decades (Omori *et al*., 1969; Savini *et al*., 2011; Eschbaumer *et al*., 2012; Breard *et al*., 2013). Disease in sheep (*Ovis aries*) is rarely reported (Uren, 1986; Yavru *et al*., 2014). EHDV outbreaks have predominantly been described in North America, Australia, and Japan. However, over the last two decades, EHDV in cattle has been increasingly reported worldwide with EHDV serotypes 1, 6, and 7 having been notified in northern Africa and the Middle East (Golender *et al*., 2019; Yadin *et al*., 2008; Temizel *et al*., 2009; Golender *et al*., 2017).

Once infected with EHDV, cattle may display bluetongue-like clinical signs, including fever, hemorrhages and erosions of the oral and nasal mucosa, crusty muzzle, drooling, swollen and reddened conjunctiva, swollen tongue with cyanosis, lameness due to coronitis, edema of the head, respiratory distress, and eventually death (Savini *et al*., 2011).

In September 2021, after a 6-year period without any official reported cases, a novel strain belonging to EHDV-8 (EHDV-8/17 TUN2021) was identified in Tunisia (Sghaier *et al*., 2022; Thabet *et al*., 2023), with an official total number of 176 cases in cattle and 2 cases in free-ranging red deer (*Cervus elaphus barbarus*) reported to WAHIS (https://wahis.woah.org/#/event-management - last accessed on 26 June 2023).

In November 2022 the same EHDV-8 strain was confirmed in the islands of Sardinia and Sicily (Italy), (Lorusso *et al*., 2023) in symptomatic cattle. This represents the first-ever official report of EHD in Europe. The outbreaks primarily involved not only cattle, but also sheep as a large sheep flock in the municipality of Fluminimaggiore (province of Sud Sardegna, Sardinia) showed an unexpectedly high number (~60%) of EHDV positive animals by molecular tests (WAHIS report EHDV_2022_16 (ob_115582). These findings raised concerns about the role of sheep in EHDV-8 epidemiology. A few days later, EHDV was also reported in Andalusia, Spain, in July 2023 in Portugal (source: https://wahis.woah.org/; last accessed on 21 July 2023), and in September 2023 in France (source: https://wahis.woah.org/; last accessed on 13 October 2023).

EHDV-8 likely reached Europe through wind-borne dissemination of infected *Culicoides* as described in the past for BTV (Cappai *et al*., 2019; Lorusso *et al*., 2017; Lorusso *et al*., 2014), a hypothesis strongly supported by the high nucleotide sequence identity (>99.9%) between the EHDV-8 strains isolated in Italy and in Tunisia across their whole genome sequences (Sghaier *et al*., 2022; Lorusso *et al*., 2023; Thabet *et al*., 2023).

The emergence of EHDV poses new challenges to the livestock industry in Europe, as currently no authorized vaccines are available, and existing legislation imposes trade restrictions of domestic live cattle and small ruminants from infected areas (Commission Delegated Regulation (EU) 2020/688 of 17 December 2019.

The situation is further complicated by the limited knowledge about the occurring EHDV-8 strain, whose first and last evidence in the field of a virus belonging to the same serotype dates to 1982 in Australia (Uren, 1986; St George *et al*., 1983). As a result, crucial information about EHDV-8 biology, including reservoir species, duration of infectious viremia, and immune response, is lacking. These knowledge gaps undermine the establishment of science-driven control policies, emphasizing the need to gather more information.

To this end, we conducted experimental infections of domestic ruminants including cattle, sheep, and goats (*Capra aegagrus hircus*) with the recently emerged EHDV-8 isolated from symptomatic cattle in Italy. Kinetics of viremia, neutralizing immune response, clinical and pathological findings are described and discussed.

Materials and methods

Ethic statement

The experimental protocol was reviewed by

the Animal Care and Welfare Board of IZSAM and authorized by the Italian Ministry of Health (authorization no. 142/2023-PR), pursuant to the Implementation of Directive 2010/63/EU on the protection of animals used for scientific purposes.

Animals

A total of six 4-5 months-old Holstein-Friesian calves (2 males and 4 females; C1 to C6), seven adult female sheep (S1 to S7), and seven female adult goats (G1 to G7) were recruited for the study. Prior to infection, animals were allowed a 7-day acclimatization period. All animals were clinically healthy and verified as free for EHDV antibodies and EHDV RNA at the time of inoculation using a competitive ELISA and a quantitative real-time RT PCR, respectively.

Four calves (C1 to C4), 5 sheep (S1 to S5), and 5 goats (G1 to G5) were randomly assigned to the infection groups, while the remaining animals served as sham-inoculated controls.

All animals were held in insect-proof stables, with each species allocated to a separate pen; water and feed were available *ad libitum*. Insecticides/insect repellents were regularly used for treating animals and the environment, and an automatic dispenser of pyrethroids operated throughout the experiment. Two black light traps were set up inside the stables and were checked three times a week for the presence of *Culicoides* insects.

Virus

An EHDV-8 strain (IZSAM internal ID 2022TE50459, EHDV-8 2022.TE.50459.1.2) was isolated through one single passage on BSR cells (a clone of Baby hamster kidney cells; RRID: CVCL_RW96) from a blood sample of a cattle collected during the EHD outbreak of 2022 in Sardinia, Italy. The infected tissue culture was harvested at 80% cytopathic effect, titrated by endpoint (titer 6.25 $\log_{10}$ $TCID_{50}$/mL), and stored at +4 °C until use. The virus inoculum was also sequenced by NGS following standard procedures already described by our group (Sghaier *et al*., 2023; Marcacci *et al*., 2016). The obtained sequence was 100% identical to that of the same strain available online (GenBank accession number OP897266-OP897274).

Animal inoculation and sampling

On day post-infection (dpi) 0, each animal in the infection groups received 2 mL of inoculum subcutaneously (1 mL for each side of the neck) and one further mL was given intradermally (left side of the neck) for a total volume of 3 mL with a concentration of 6.2 $\log_{10}$ $TCID_{50}$/mL.

Control animals were sham inoculated with the same volume of cell culture medium at the same injection points. Starting from 1 dpi and up to 14 dpi, animals were manually restrained once a day for clinical examination. Body temperature was measured rectally from -3 dpi to 14 dpi. The mean of the temperatures measured from -3 dpi to 0 dpi was considered as the basal temperature. An increase of ≥ 1°C from 1 dpi onward was considered as fever.

Blood samples were collected by jugular venipuncture in EDTA and plain tubes three times a week from -7 dpi to 39 dpi (including dpi 0), twice a week from 43 dpi to 60 dpi, and once a week from 64 dpi to 78 dpi (end of the experiment). Goats were sampled up to 46 dpi.

After blood clotting in plain tubes, serum was separated and stored at +4 °C until analysis. EDTA-blood samples were used for EHDV-8 RNA detection, virus isolation, and complete blood count (including platelet count); serum samples were used for serology (ELISA and VN), and hematochemical analysis.

Real-time RT PCR

Total RNA was extracted from the blood and organ samples using the KingFisher™ Flex 96 platform (Thermo Fisher Scientific, MA, USA), with the MagMAX™ CORE Nucleic Acid Purification Kit (Thermo Fisher Scientific, Waltham, MA, USA), according to the manufacturer's instructions. Purified nucleic acids were tested for the presence of EHDV RNA using an in-house serogroup-specific real time RT-PCR (herein after, qPCR). The qPCR was designed to detect a portion of S9 of all known EHDV serotypes (Maan *et al*., 2017).

The assay was optimized using TaqManTM Fast virus 1-step Master Mix (Thermo Fisher Scientific, MA, USA), with primers and a probe at concentrations of 600 nM and 200 nM, respectively, in a reaction volume of 25 µL. VetMAXTM Xeno™ Internal Positive Control RNA (Thermo Fisher Scientific) was included as an exogenous process control. Amplification was carried out using QuantStudio™ 7 Flex Real-Time PCR System (Thermo Fisher Scientific) with the following thermal cycles: 55°C for 30 min, 95°C for 10 min followed by 50 cycles of 95°C for 30 s and 60°C for 1 min.

Virus isolation

Virus isolation was attempted from all qPCR-positive EDTA-blood samples by means of a first passage on BSR cells (RRID: CVCL_RW96) cells or on the *Culicoides sonorensis* cell line (KC cells; RRID: CVCL_RW99) (blind passage), followed by a second passage on BSR cells only.

For the BSR inoculum, 0.5 mL of EDTA-blood was suspended in phosphate-buffered saline (PBS) and centrifuged at 800 rpm for 2 minutes. The red blood cell pellet was washed twice with 0.5 mL PBS and then resuspended in 0.5 mL of distilled water to allow cell lysis and virus release.

Lysed blood (0.2 mL) was then inoculated on a preseeded (3 hrs at 37 °C) BSR cells monolayer and incubated for 2 hrs at 37 °C with 5% CO_2. The inoculum was then replaced with 1 mL of minimum essential medium (MEM - Sigma-Aldrich, St. Louis, Missouri, USA) containing 10% foetal calf serum.

After 5 days of incubation at 37°C, the supernatant from the infected cell monolayers was collected and the cell monolayers were detached by trypsin and versene treatment.

The detached cells were mixed with 1mL of the retained supernatant, and 0.2 mL of the mixture was transferred to a new preseeded BSR cell monolayer (2^{nd} passage) and incubated as described before. Cultures were monitored daily for 5-7 days.

A cell culture was considered negative if cytopathic effect (CPE) was not observed after the second passage. After CPE development, cells were scraped off, and the success of viral isolation was confirmed by qPCR.

For propagation on KC cells, pre-seeded (24 hrs at 28 °C) monolayers were infected with 0.2 mL of the lysed blood samples and incubated for 2 hrs at 28°C. The monolayers were then washed with serum-free medium and incubated for 10 days with Schneider's insect medium (Sigma-Aldrich, St. Louis, Missouri, USA) containing 3% fetal calf serum. The cells were subsequently scraped off, and the cell pellet obtained after centrifugation was inoculated on a pre-seeded (3 hrs) BSR cell monolayer for 5–7 days (2^{nd} passage).

After cytopathic effect (CPE) was evidenced on BSR cells (2nd passage), cells were scraped off, and viral isolation was confirmed by qPCR and/or immunofluorescence.

End-point virus titration of isolates obtained from the correspondent blood samples was performed using BSR cells, starting from 1 $\log_{10}$ dilution of the blood. The end-point titer was calculated using the method of Reed and Munch (1938).

Serology

Antibodies to EHDV VP7 in serum samples were detected using a commercial competitive ELISA kit (cELISA, ID Screen EHDV competition; Innovative Diagnostics, Grabels, France), following manufacturer's instructions. This assay has a reported specificity of 100% (IC 95%: 99.7–100) and a sensitivity of 100% (IC 95%: 96.76-100).

The results are expressed as the competition percentage (S/NC%), using the following formula: OD of the sample/OD of the negative control) x100. Samples with S/NC% values ≥ 40 are classified as negative, values > 30 and < 40 are considered as doubt, and values ≤ 30 are classified as positive.

Neutralizing antibodies were titrated by virus-neutralization test as described in the dedicated chapter of the WOAH Manual of diagnostic tests and vaccines for terrestrial animals (https://www.woah.org/fileadmin/Home/eng/Health_standards/tahm/3.01.07_EHD.pdf - last accessed 07 June 2023), using the EHDV-8/17 TUN2021 isolate (Sghaier *et al*., 2023).

The neutralizing antibody titer was defined as the reciprocal of the highest dilution of the tested serum able to neutralize 100% CPE in the wells.

Hematological analyses

Hematological parameters were evaluated only in cattle. All hematological analyses were conducted within 24 hrs after blood collection. Serum electrophoresis was performed on cellulose acetate membranes using commercial kits (Auto Phor 400 – Biogroup Medical System, Rimini - Italy), following the manufacturer's instructions. After sample deposition, all operative phases were performed by the automated electrophoresis analyzer Selvet 24 (Seleo Engineering s.r.l., Caserta, Italy). Blood chemistry parameters, including blood urea nitrogen, creatinine, total protein, albumin, alkaline phosphatase, aspartate-transaminase, and alanine-aminotransferase, were evaluated using commercial kits (Quantilab Kits, Werfen Company, Milano, Italy) and an ILAB 650 automated system (Instrumentation Laboratory, MA, USA). White blood cells, red blood cells, hemoglobin concentration, platelet count, and hematocrit were assessed using the ADVIA 2120i Hematology System (Siemens, Erlangen, Germany), following the manufacturer's instructions.

Statistical analysis

A non-parametric Mann-Whitney test was applied to assess the differences in hematological parameters, before and after EHDV-8 inoculation. All statistical analyses were carried out using the R software (R Core Team, 2022). Differences were considered significant when the p-value was less than 5% ($p< 0.05$).

Necropsy

At 80 dpi and 87 dpi, two EHDV RNA-positive sheep (S1 and S4) and four EHDV RNA-positive cattle were (C1 to C4) were humanely euthanized and inspected for gross lesions.

A portion of the spleen, liver, lung, and kidney was excised and tested by qPCR.

Results

EHD clinical signs were observed in cattle and sheep

Following experimental, infection, three inoculated calves (C1, C2, and C4) showed fever which lasted for one day (6 dpi). Starting at 6 dpi, C4 displayed clinical signs consistent with EHD, including reddening, ulcers, and hemorrhages of the nostrils (Figure 1A). These lesions progressively worsened, resulting in crusty lesions and ulcers covering a significant portion of the muzzle (Figure 1B). Mild drooling was also observed, although no oral lesions were present. At 10 dpi, following the sloughing of the crusts, ulcers and haemorrhages were observed (Figure 1C). S1 showed fever at 8 and 9 dpi. In contrast, goats and the sham-inoculated animals did not show any clinical signs throughout the entire experimental period.

Infectious virus was recovered from cattle and sheep

At 2 dpi, EHDV RNA was detected in the blood samples of C2 and C3 (Ct 38.1 and 36.7, respectively). From 4 dpi onwards, all infected calves consistently tested positive for EHDV RNA. Ct values (a reliable proxy for EHDV RNA levels, the lowest Ct the highest virus magnitude) were the lowest at 7 dpi (Ct range 19.1-25.8) for all animals; subsequently, the values gradually increased but remained relatively stable from 25/28 dpi until the end of the trial at 78 dpi (Ct range 26.9-34.4) (Figure 2A).

Infectious virus was isolated from the blood of C2, C3, and C4 at 7 and 9 dpi; from all calves at 11 dpi; from C2 and C3 at 16 dpi, and from C3 at 21 dpi (Table I). EHDV RNA was detected in S1, S3, and S4 (Ct 33.3, 38.0, and 32.8, respectively) at 2 dpi. However, only two individuals (S1 and S4) remained RNA-positive until the last day of sampling (78 dpi - Ct values 31.3 and 37.8, respectively), with the lowest Ct at 7 dpi for both animals. In the remaining sheep, RNA was detected up to 11 dpi (Figure 2B). Infectious virus was recovered from S1 and S4 at 4 dpi, and from S1 at dpi 7 (Table II). All isolates were obtained only by means of the combination of KC and BSR cells. Titration of the virus straight from infected blood samples was consistently unsuccessful (limit of detection of the method < 1.3 $\log_{10}$ $TCID_{50}$/mL).

All goats and sham-inoculated animals tested negative for EHDV RNA throughout the entire experimental period (Figure 2C).

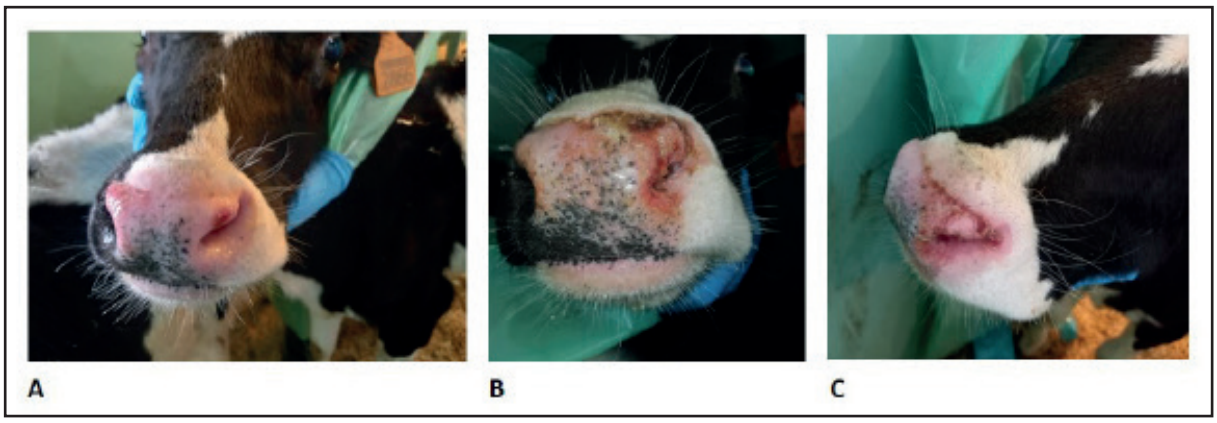

Figure 1. *Starting at 6 dpi, C4 displayed clinical signs consistent with EHD infection including reddening, ulcers, and hemorrhages of the nostrils (A). These lesions progressively worsened, resulting in crusty lesions and ulcers covering a significant portion of the muzzle (B). Mild drooling was also observed, although no oral lesions were present. At 10 dpi, following the sloughing of the crusts, ulcers and haemorrhages were observed (C).*

Table I. *Infectious virus was recovered from the blood samples of all infected calves. Ct, threshold cycle; Isol., isolation. +, positive; -, negative.*

	Day post-infection									
	7		***9***		***11***		***16***		***21***	
Calf ID	Ct	Isol.	Ct	Isol.	Ct	Isol.	Ct	Isol.	Ct	Isol.
C1	25,8	-	26,4	-	***28,8***	+	31,0	-	33,0	-
C2	***19,2***	+	***20,6***	+	***22,4***	+	***23,9***	+	24,4	-
C3	***19,1***	+	***19,4***	+	***20,9***	+	***22,9***	+	***23,9***	+
C4	***21,0***	+	***22,4***	+	***24,8***	+	26,2	-	26,3	-

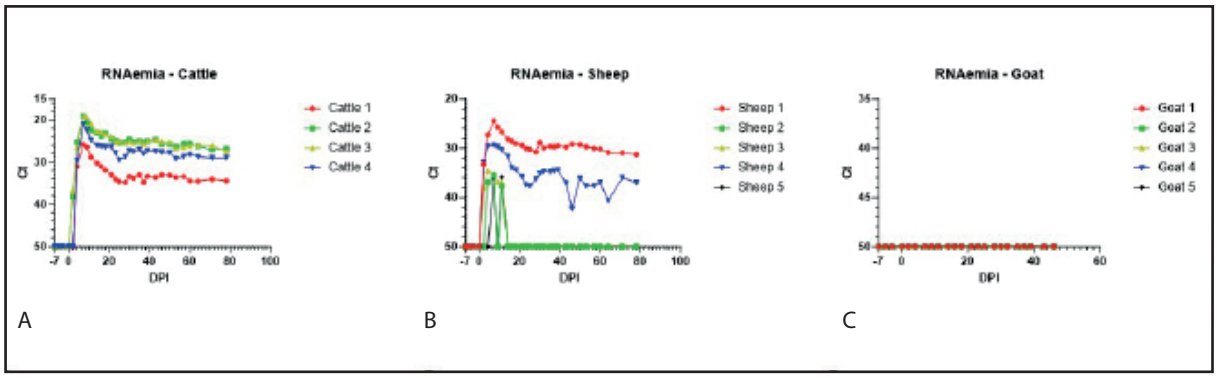

Figure 2. *Kinetics overtime of EHDV-8 RNA Ct values in the blood samples of cattle (A), sheep (B), and goats (C). x-axis: day post-infection (dpi); y-axis: Ct cycle threshold (Ct).*

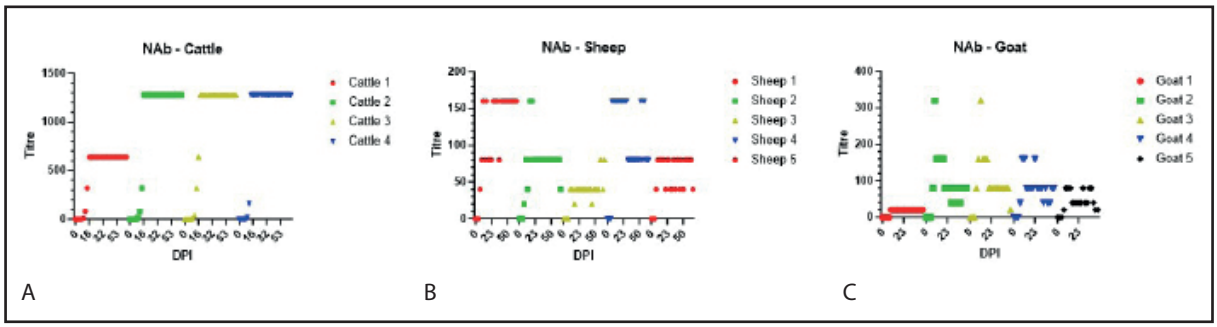

Figure 3. *Kinetics of EHDV-8 neutralizing antibody titer in calves (A), sheep (B), and goats (C) over time. x-axis: day post-infection (dpi); y-axis: neutralizing antibody titer. The neutralizing antibody titer is defined as the reciprocal of the highest dilution of the serum capable of neutralizing 100% cytopathic effect.*

Table II. *Infectious virus was recovered from the blood samples of two infected sheep. Ct, threshold cycle; Isol., isolation. +, positive; -, negative.*

	Day post-infection			
	4		*7*	
Sheep ID	Ct	Isol.	Ct	Isol.
S1	***27,3***	+	***24,5***	+
S2	37,0	-	35,6	-
S3	34,6	-	35,3	-
S4	***29,6***	+	29,4	-
S5	50,0	-	36,2	-

All infected animals showed robust neutralizing antibodies response

Seroconversion, as determined by cELISA, was observed in all calves from 9 dpi onwards.

All sheep and goats seroconverted by 9 dpi and remained cELISA-positive until the end of the sampling period (dpi 78 and dpi 46 for sheep and goats, respectively). Furthermore, all infected animals developed a neutralizing immune response starting from 9 dpi. Calves showed significantly higher neutralizing titers compared to small ruminants, with a highest titer of 1280 (upper limit of detection for virus-neutralization) detected from 14 dpi (Figure 3A).

In contrast, the neutralizing titers in sheep were, overall, lower than cattle (Figure 3B). In goats, despite the lack of detectable viral replication, a robust neutralizing antibodies response was also evidenced, except for one goat that exhibited a weaker response (highest titer of 10, Figure 3C). Sham-inoculated animals tested constantly negative for EHDV antibodies during the entire trial.

Red and white blood cell count differed significantly before and after infection

Red blood cell count decreased significantly following infection (median pre-infection 8.905 $x10^6$/mcl vs. median post-infection 8.065 $x10^6$/mcl; $p<0.001$), while white blood cell count increased significantly (median pre-infection 10.09 $x10^3$/mcl vs. median post infection 11.87 $x10^3$/mcl; $p=0.04$). The other parameters did not show any significant difference before infection compared to post infection (Table III, Figure 4).

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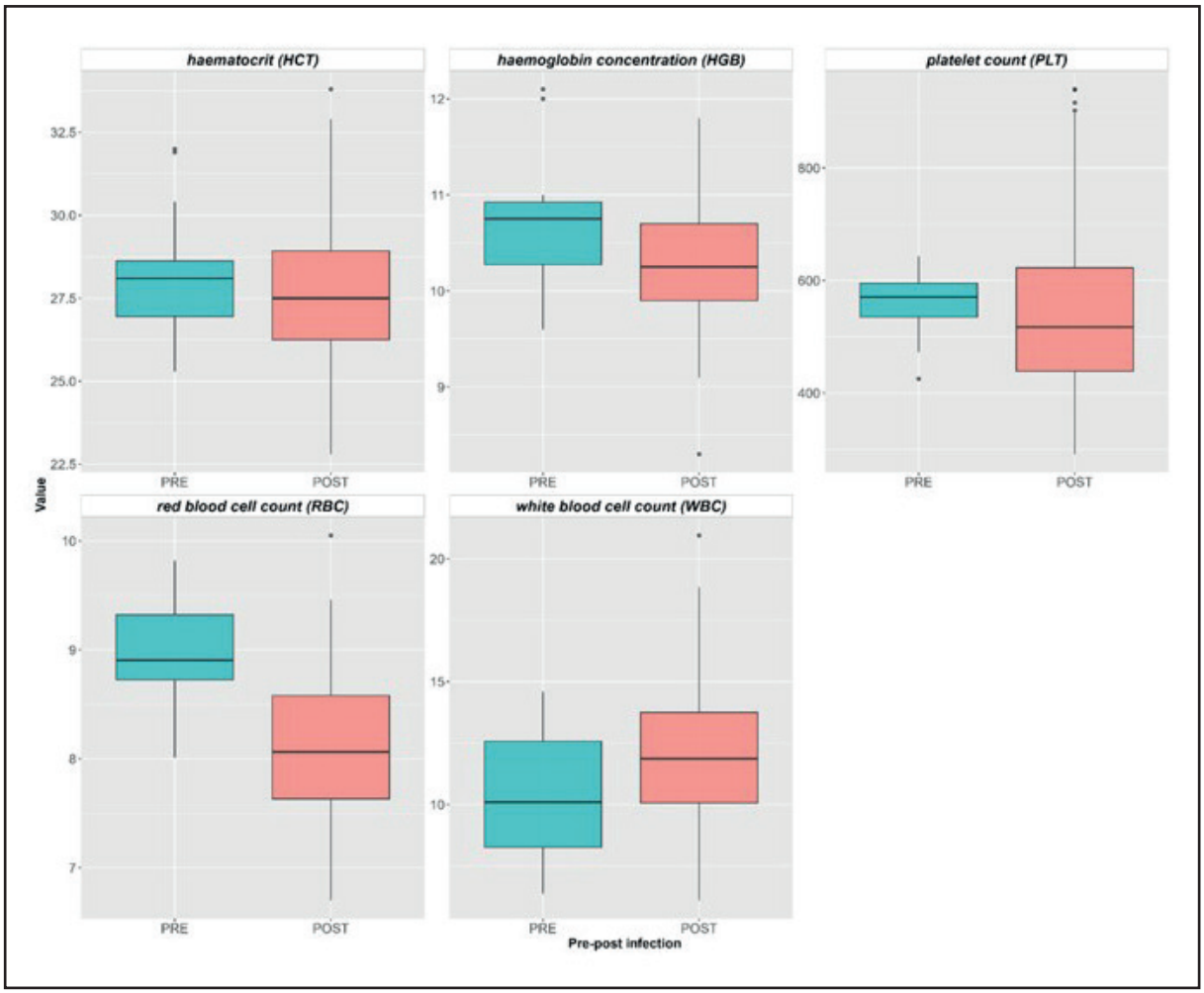

Figure 4. *Box plots: data distribution of each blood parameter, pre and post infection.*

Table III. *Comparison between values of the haematological parameters before and after infection in calves. A value of p<0.05 indicates significant difference (bold).*

Parameter	median pre-infection	median post-infection	P value
haematocrit (%)	28.1	27.5	0.456
haemoglobin concentration (g/dL)	10.75	10.25	0.053
platelet count (10^3/mcL)	570.5	517	0.288
red blood cell count (10^6/mcL)	8.905	8.065	**<0.001**
white blood cell count (WBC) (10^3/mcL)	10.09	11.875	**0.04**

Specific gross lesions were not evidenced

No gross lesions were observed at necropsy in any of the animals. The spleens of sheep S1 and S4 tested positive for EHDV RNA by qPCR (Ct 33 and 35, respectively).

The lungs of calves C2 and C3, and the spleens of calves C3 and C4, tested positive by qPCR, with Ct 37 and 36 for the lungs and 31 and 30 for the spleens, respectively. Isolation of infectious virus, however, was unsuccessful.

Discussion

In this experiment, we described, for the first time, the results of an experimental infection with the recently emerged EHDV-8 in domestic ruminant species, including cattle, sheep, and goats.

To the best of our knowledge the only documented experimental infection of sheep and cattle with EHDV-8 dates to 1986 (Uren, 1986). In that study, the Australian prototype strain of EHDV-8 was inoculated intravenously in sheep and calves. In that case, none of the cattle exhibited clinical signs despite the presence of viremia. In contrast, in this trial, three out of four infected calves exhibited fever for one day. In addition, one calf developed overt clinical signs including ulcerative and crusty lesions on the muzzle.

In calves, viral RNA levels peaked at dpi 7, coinciding with the onset of clinical signs. Along with the presence of high levels of neutralizing antibodies, the viral RNA levels gradually declined but remained relatively stable throughout the experimental period. This observation is consistent with field observations where qPCR-positive results were obtained from sequential blood samples of the same animals up to 5 months following the first diagnosis (Lorusso, personal communication). The recovery of infectious virus from the blood was anyway possible only for a shorter period, from dpi 7 up to dpi 21. Indeed, qPCR-positive results for viral RNA do not necessarily indicate the presence of infectious virus, as seen in other closely related viruses such as BTV.

Various studies have provided insights into the duration of infectious viremia for different EHDV serotypes in cattle. For example, Gibbs and Lawman (1977) documented that EHDV-2 could be isolated from calves for up to 28 days after infection, while viremia was observed from day 9 to day 23 for EHDV-1 and from day 8 to day 16 for a different EHDV-2 strain (Aradaib *et al*., 1994). Another study reported viremia lasting up to day 44 in a calf infected with a further EHDV-2 strain (Abdy *et al*., 1999), while experimental infection with EHDV-7 resulted in the detection of infectious virus up to 18 days post-infection (Ruder *et al*., 2015). As for EHDV-8, natural cases typically indicate that viremia lasted less than a week in most animals (Gard and Melville, 1992), while Uren (1986) reported a mean viremia duration of 10 days (range 9-13) following infection with the Australian prototype strain of EHDV-8.

As far as sheep are concerned, our trial showed that one out of five infected sheep, experienced a 2-day fever. One of the most important findings of this study is the evidence of infectious viremia in sheep. While the presence of EHDV RNA was transient in four animals but persistent (up to 78 dpi) in two of them, isolation of infectious virus was possible up to 7 dpi from one sheep. This could reasonably suggest a role of sheep in the epidemiology of this newly emerged Orbivirus. This is in stark contrast to previous observations indicating that sheep have a negligible role in EHDV epidemiology (Kedmi *et al*., 2011). Our experimental evidence couples with an in-field observation in a sheep flock located in the municipality of Fluminimaggiore, Sardinia, where 67 out of 111 susceptible sheep tested positive for EHDV-8 by qPCR. In that case, however, attempts to isolate the virus were unsuccessful (Ciriaco Ligios *et al*., personal observation).

Regarding the serological response, our results demonstrate the development and persistence of high titers of serotype-specific neutralizing antibodies. This suggests that in field conditions animals could be protected against reinfection with a homologous serotype for an extended period. However, the exact duration of this protection cannot be determined based on our study alone, and further research is needed.

Taken together, experimental and field observations, though limited up to now in Italy, could indicate sheep as potential contributors to the transmission and maintenance of EHDV-8, particularly in regions with a high sheep population density, such as Sardinia. Goats consistently tested negative by qPCR during the entire course of the trial suggesting that contribution of goats to the transmission of the virus may be considered negligible. However, similar to cattle, sheep and goats also mounted a neutralizing immune response, although at a lower magnitude. The variation in neutralizing titers between calves and small ruminants is not surprising, and may be attributed to differences in host susceptibility.

We also investigated the haematological parameters in cattle following infection with EHDV-8 as literature data are limited to white-tailed deer. The significant decrease in RBC count post-infection could be a common feature in calves infected with EHDV-8, though anaemia was not clinically evidenced in this study. On the other hand, the significant increase in WBC following infection could indicate the activation of the immune system in response to EHDV-8 infection in the animals.

In conclusion, our experimental study demonstrates the clinical, laboratory and epidemiological impact of EHDV-8 in domestic ruminants. Moreover, it provides important information on the kinetics of EHDV-8 in cattle and sheep, providing crucial hints to policy makers for designing effective control and prevention strategies to mitigate the impact of this viral disease in livestock.

The results demonstrate the ability of the newly emerged EHDV-8 to infect small ruminants and cattle, and to replicate efficiently in cattle and sheep, with variations in clinical manifestations and viral dynamics. While the role of cattle as reservoirs for the virus is well-known, the potential role of sheep has been scarcely investigated. Here, we suggest that sheep can indeed be considered a potential reservoir for EHDV-8, also considering the in-field observation in the sheep flock located in Fluminimaggiore.

Further research with larger sample sizes and real-world observations are warranted to fully understand the extent of sheep involvement in EHDV-8 epidemiology. Nevertheless, this information must be considered for the development of disease management and surveillance strategies, and control tools, especially in regions with dense sheep populations.

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