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Emergence of epizootic hemorrhagic disease in red deer (*Cervus elaphus*), Spain, 2022

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ABSTRACT

Epizootic hemorrhagic disease (EHD) virus serotype 8 (EHDV-8) emerged in Spain in autumn 2022. In this study, we aimed to (1) characterize the clinical and lesional presentation of EHDV infection in European red deer (*Cervus elaphus*), and (2) study the spatial spread of the virus in wild ruminants in Spain after its introduction, in 2022/2023. We confirmed EHDV infection in two clinically compatible sick red deer by PCR and detection of anti-EHDV specific antibodies. EHDV infection occurred in red deer with hyperacute to acute clinical signs and lesions associated to vascular changes leading to death of the animals. Partial sequences of variable segment 2 (VP2) and segment 5 (NS1) genes of the detected viruses had >99% nucleotide identity with EHDV-8 sequences from Tunisia and Italy. In a cross-sectional serological study of EHDV in 592 wild ruminants, mainly red deer (n=578), in southwestern Spain, we detected anti-EHDV antibodies in 37 of 592 samples (6.3%; 95% confidence interval: 4.3–8.2), all from red deer and from the localities where clinical cases of EHD were confirmed in red deer. We conclude that EHDV-8 infection causes severe EHD in European red deer. The serosurvey revealed a limited spread of EHDV-8 in Spanish wild ruminant populations in the first year of virus detection in Spain.

1. Introduction

Epizootic hemorrhagic disease (EHD) is a reportable vector-borne disease of wild and domestic ruminants caused by an *Orbivirus* related to bluetongue virus (BTV). Seven EHD virus (EHDV) serotypes are known, distributed over five continents (Jiménez-Cabello et al., 2023) and with a variable pathogenic potential (EFSA, 2009). Variants of serotypes 1, 2 and 6, that are enzootic in North America, have caused severe EHD outbreaks with high mortality in white-tailed deer (*Odocoileus virginianus*) and the loss of 1.7% of farmed cervids in the United States between 2013 and 2014 (USDA, 2016). EHDV can also affect other wild ruminants such as mule deer (*Odocoileus hemionus*), pronghorn antelope (*Antilocapra americana*), elk (*Cervus canadensis*) (USDA, 2016) and Barbary deer (*C. elaphus barbarus*) (Thabet et al., 2023).

In 2006, an epidemic of EHDV serotype 6 (EHDV-6) spread across Morocco, Algeria, and Tunisia, and entered the European continent in 2007 with the notification of EHD cases in cattle in Turkey (EFSA, 2009). However, in contrast to what it was observed with BTV serotypes 1 and 4 after their introduction into Spain in 2004 and 2007, respectively (Ruiz-Fons et al., 2014) and with BTV serotype 8 in west-central Europe

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in 2008 (van Rijn et al., 2012), EHDV-6 did not spread from North Africa and Turkey to neighboring European countries. In 2021, more than 200 EHD outbreaks were notified in cattle in Tunisia caused by EHDV serotype 8 (EHDV-8) (Sghaier et al., 2023). In autumn 2022, the first EHDV-8 cases were reported in cattle in Italy (Sicily and Sardinia; Lorusso et al., 2023) and in southwestern Spain (Jiménez-Cabello et al., 2023). Almost simultaneously with the reporting of EHD outbreaks in cattle in autumn 2022, passive wildlife disease surveillance detected in southwestern Spain outbreaks of unusual mortality and isolated clinical cases in native red deer (*Cervus elaphus*). Our objectives were (1) to confirm whether the fatal cases observed in red deer were EHD and (2) to determine the spread of the virus among wild ruminants during its first incursion into Spain.

2. Methods

2.1. Clinical cases in red deer

In mid-November 2022, we were notified of an unusual outbreak of mortality in a red deer farm in southwestern Spain (Fig. 1). On November 19, 2022, we performed a necropsy on a 1.5-year-old male deer from that farm (Case 1) which had been observed with depression, anorexia, weakness, ataxia, and severe respiratory distress three days before being found dead. On November 28, 2022, during a rutinary visit to an integrated wildlife monitoring site 180 km North of Case 1, we found a moribund stag presenting severe dyspnea and weakness (Case 2). The animal died within an hour of detection and was necropsied. Organ samples (lung, spleen, kidney, liver, heart) were fixed in 10% phosphate-buffered formalin, embedded in paraffin, sectioned at 4 µm

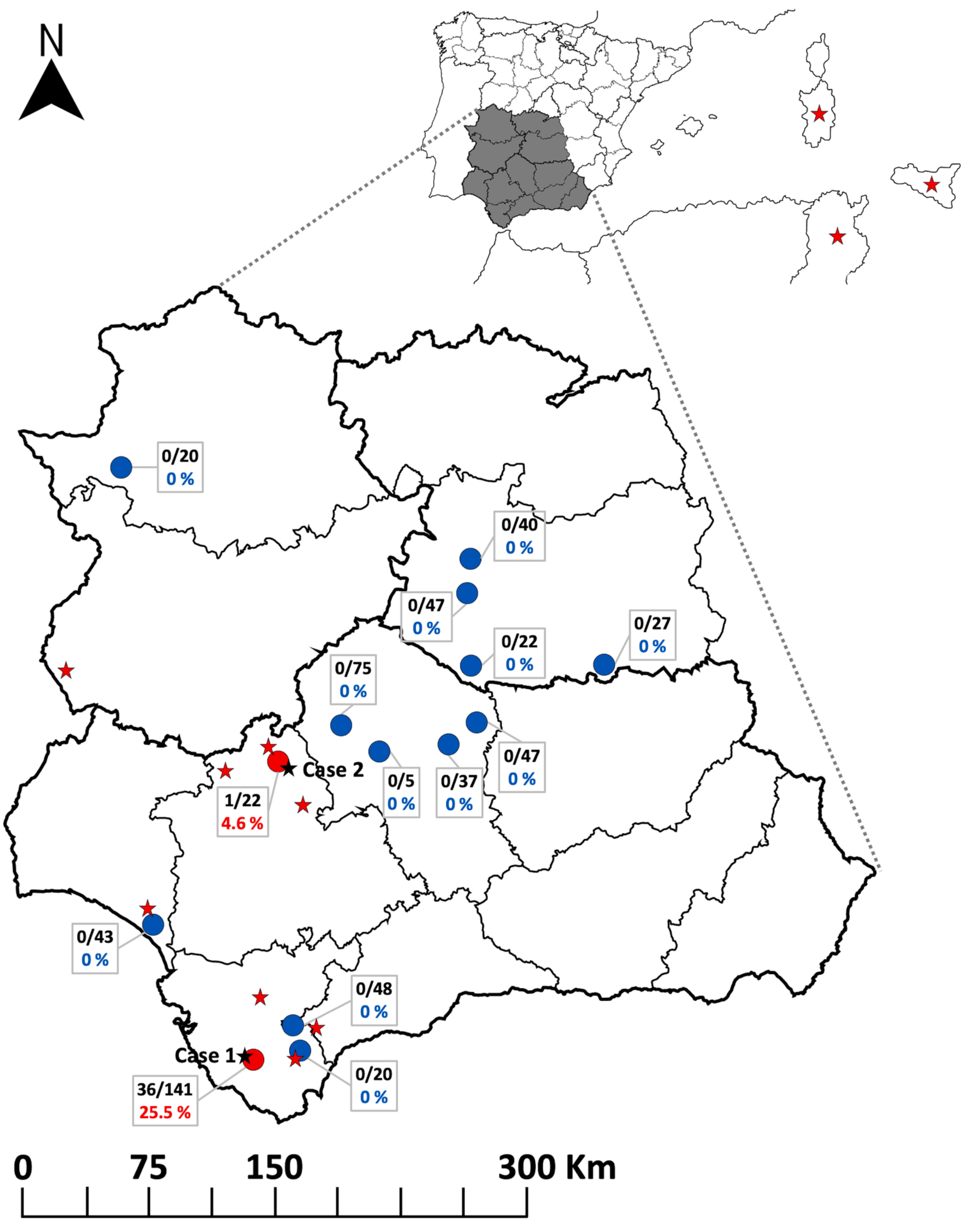

Fig. 1. Map of southwestern Spain (bottom left) and its location within the western Palearctic (top right) showing the spatial distribution of the 14 wild ruminant populations studied within provincial boundaries (black lines) and serological results (blue circle: seronegative; red circle: seropositive). Positive animal samples in relation to those tested (positive/sample size) and seroprevalence (in %) per population are shown. The red stars indicate the countries/localities where outbreaks of epizootic hemorrhagic disease in cattle were reported in 2022 (MAPA, 2023), while the black stars show the location of the two clinical cases in red deer with specification of the case number.

and stained with hematoxylin and eosin.

Samples of spleen and blood taken at necropsy were subjected to RNA extraction (NucleoSpin RNA, Macherey-Nagel, Germany). The presence of EHDV in all tissue samples was tested using a real-time RT-PCR duplex pan-EHDV protocol, which targets the segment 9 gene of EHDV coupled with a real-time RT-PCR that amplifies ruminant beta-actin RNA (Viarouge et al., 2015), in a CFX96 Touch Deep Well™ Real-Time PCR Detection System (Bio-Rad, CA, USA). To further characterize the virus, we amplified and sequenced a 558 bp gene fragment of the variable segment 2 (VP2) and a fragment of the segment 5 (NS1), both highly expressed and conserved viral regulatory proteins. VP2 and NS1 genes were partially amplified by RT-PCR in positive samples using in-house primers selected based on the published sequence of EHDV-8 from Tunisia (Sghaier et al., 2023) and a previously described protocol (Aradaib et al., 1994), respectively. For NS1, we amplified and sequenced a 225 bp fragment in Case 1 and a 348 bp fragment in Case 2. The obtained sequences were deposited in the GenBank database and compared with other previously reported EHDV sequences (Accession numbers are shown in Table 1).

2.2. Cross-sectional serological survey

Between November 2022 and February 2023, we collected blood samples from red deer (n=578), fallow deer - *Dama dama* (n=3), and mouflon - *Ovis orientalis musimon* (n=11) in 14 localities of southwestern Spain, both in areas of confirmed EHD cattle cases (MAPA, 2023) and in neighboring areas (Fig. 1). Samples were obtained from animals shot by rangers and hunters during population control and commercial hunting events, respectively. Cases 1 and 2 were also included in the serosurvey. We searched for specific antibodies against EHDV VP7 in sera obtained from blood samples using a commercial multi-species competition ELISA (ID Screen® EHDV Competition, ID.vet, France; Bréard et al., 2020).

3. Results

3.1. Clinical cases

On external inspection, Cases 1 and 2 presented poor body condition, severe congestion and cyanosis of the oral mucosa and tongue (Fig. 2A).

Table 1
Sequence data of variable segment 2 (VP2) and segment 5 (NS1) of epizootic hemorrhagic disease (EHD) virus serotype 8 obtained from two clinical cases of EHD in European red deer (*Cervus elaphus*) confirmed in 2022 in Spain.

Segment	Reference	GenBank Accession No	EHDV-8 sequences compared (Accession No)	Identity (%)
VP2	Case 1	OR188849	OP381191, OP897266, OP897276, OP897501, OP897511, OP897521, OP897531, OP897541, OP897551, OP897561, OP937332, OP971118, OP971128, OP971138	100
	Case 2	OR188850	OP381191, OP897266, OP897276, OP897501, OP897511, OP897521, OP897531, OP897541, OP897551, OP897561, OP937332, OP971118, OP971128, OP971138	99.5
NS1	Case 1	OR188846	OP971141, OP971131, OP971121, OP937335, OP897564, OP897554, OP897544, OP897534, OP897524, OP897514, OP897504, OP897279, OP897269, OP381194	100
	Case 2	OR188847	OP971151	99.6

Both showed slight pulmonary congestion and presence of liquid and froth in the trachea and bronchi, compatible with a severe alveolar edema (Fig. 2B). One of them also presented a moderate number of petechial and ecchymotic hemorrhages in the parietal pleura (Case 1), while the other animal showed an ulcerative lesion on one ear.

Microscopically, the examination of lungs revealed severe alveolar and interstitial edema along with light congestion (Fig. 2C). Lymphoplasmacytic vasculitis on medium to large vessels was observed in lung, heart, and liver (Fig. 2D, E). Glomerular capillaries of the kidneys were congested. Lymphoid follicles of the spleen showed depletion of lymphocytes (Fig. 2F). Thrombi were not seen.

We detected EHDV in all the tissues and blood collected from both deer at Ct values ranging from 22 to 29, and confirmed EHDV-8 infection in both cases. Ct values were lower in the blood than in the liver of both animals. The BLAST analysis showed high nucleotide identity for both VP2 (>99.5% among the two sequences and with available EHDV-8 sequences from Tunisia and Italy; Lorusso et al., 2023; Sghaier et al., 2023; Table 1) and NS1 gene segments (100% among the two sequences and >99.6% with reported EHDV-8 sequences; Lorusso et al., 2023; Sghaier et al., 2023).

3.2. Serological survey

We detected anti-EHDV antibodies in 37 of 592 (6.3%; 95% Confidence Interval: 4.3–8.2) wild ruminants, including Cases 1 and 2, but only in red deer and in the locations where Cases 1 and 2 occurred (Fig. 1). None of the wild ruminant samples collected at localities with confirmed EHD cattle cases (MAPA, 2023) were seropositive except Case 2 (Fig. 1).

4. Discussion and conclusions

Our findings confirm that EHDV-8 can cause clinical disease and mortality in European red deer. The two reported cases suggest a hyperacute-to-acute clinical presentation. The lesions observed were associated to vascular changes such as congestion, edema, and vasculitis. However, other typical histologic lesions caused by EHDV-2 and EHDV-6 in white-tailed deer (Rodrigues et al., 2022) or by EHDV-8 in Barbary deer (Thabet et al., 2023), such as endothelial swelling, thrombosis, fibrinoid necrosis and multiple hemorrhages, were not observed.

The serosurvey reveals a limited spread of EHDV-8 in wild ruminant populations in the first year of virus detection in Spain, even in locations where EHD was confirmed in cattle. Higher seroprevalence was observed in the southernmost of the two seropositive locations, supporting the presumed introduction of EHDV from North Africa. However, in summer 2023, EHDV-8 underwent a notable expansion in Spain causing numerous outbreaks with red deer mortality, and it appeared in Portugal and France (MAPA, 2023). The impact of the emergence of EHD on red deer population dynamics is unknown. The outbreak on the farm where we report Case 1 caused mortalities approaching 4,1%, with 10% mortality in the most affected batches of the farm. We believe that the impact of EHD emergence will be highly variable among red deer populations depending on local conditions, such as the structure of EHDV hosts and vector communities. Future studies should be carried out to fill this knowledge gap.

The wide range of competent *Culicoides* spp. vectors for EHDV is likely to favor further spread of EHDV in wild ruminants in Iberia and in other European countries in the near future. Integrated wildlife and vector population monitoring, and disease surveillance (Barroso et al., 2022) will aid to assess the spread and impact of this emerging *Orbivirus*.

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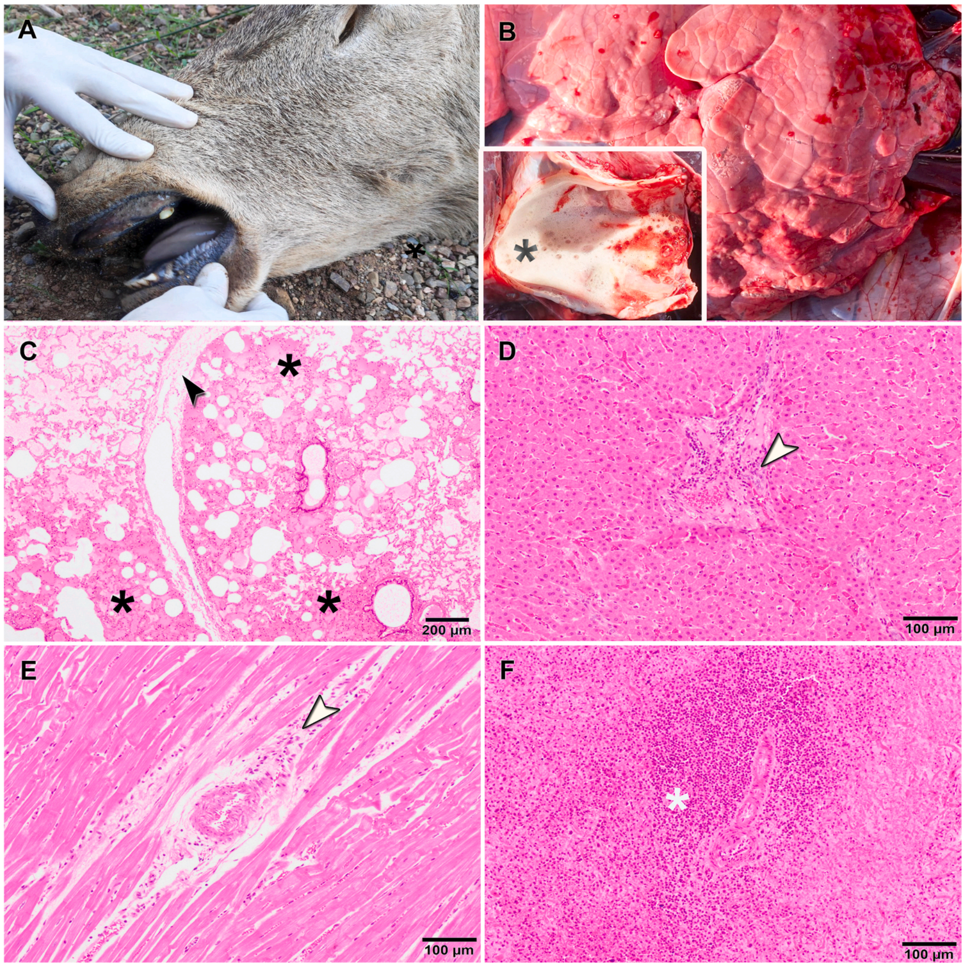

Fig. 2. Lesions consistent with emerging epizootic hemorrhagic disease in European red deer (*Cervus elaphus*). A) Severe cyanosis of the oral mucosa and tongue. B) Light congestion and increased size of the lung, observing a lobulillar-like pattern in its surface and presence of liquid and froth in the trachea and bronchi (grey asterisk, inset). C) Severe acute pulmonary edema with accumulation of fluid inside alveoli (black asterisks) and interstitium (black arrowhead). D) Portal hepatitis and myocarditis (E) associated to lymphoplasmacytic perivasculitis (white arrowheads). F) Mild depletion of lymphocytes in a lymphoid follicle of the spleen (white asterisk).

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CRediT authorship contribution statement

Christian Gortázar: Writing – review & editing, Supervision, Funding acquisition. **María Ángeles Risalde:** Writing – review & editing, Writing – original draft, Validation, Supervision, Resources, Methodology, Investigation, Funding acquisition, Data curation, Conceptualization. **Francisco Ruiz-Fons:** Writing – review & editing, Writing – original draft, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Ignacio García-Bocanegra:** Writing – review & editing, Validation, Investigation, Conceptualization. **Marta Valero:** Writing – review & editing, Methodology, Investigation, Data curation. **Raúl Cuadrado-Matías:** Writing – review & editing, Methodology, Investigation, Formal analysis, Data curation. **David Relimpio:** Writing – review & editing, Methodology, Data curation. **Remigio Martínez:** Writing – review & editing, Writing – original draft, Validation, Methodology, Data curation. **Sara Baz-Flores:** Writing – review & editing, Methodology, Data curation. **Moisés Gonzálvez:** Writing – review & editing, Visualization, Methodology. **David Cano-Terriza:** Writing – review & editing, Supervision, Data curation. **José Antonio Ortiz:** Writing – review & editing, Visualization, Data curation.

Declaration of Competing Interest

The authors declare no competing interests.

Data availability

Data are available upon request to the corresponding author.

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