

DETEÇÃO DO VÍRUS DA DOENÇA EPIZOÓTICA HEMORRÁGICA EM *CULICOIDES* CAPTURADOS EM IDANHA-A-NOVA, CASTELO BRANCO, PORTUGAL

DETECTION OF EPIZOOTIC HEMORRHAGIC DISEASE VIRUS IN *CULICOIDES* CAPTURED IN IDANHA-A-NOVA, CASTELO BRANCO, PORTUGAL

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Resumo: A Doença Epizootica Hemorrágica (DHE), transmitida a ruminantes domésticos e selvagens por fêmeas do género *Culicoides*, associa-se a elevada morbilidade, mortalidade e perdas económicas significativas. Em 2022, a doença foi detetada pela primeira vez na Europa, em Itália e no sul de Espanha. Durante um surto ocorrido em Portugal (setembro-novembro de 2023), foram recolhidos exemplares de *Culicoides* em Idanha-a-Nova, distrito de Castelo Branco, com o objetivo de avaliar a presença do vírus da DHE no vetor. O vírus foi detetado em 13 dos 19 pools de *Culicoides* analisados (68,42%), evidenciando uma prevalência elevada. A morbilidade dentro de cada rebanho variou entre 0,60% e 51,42%, com um valor médio de 11,34%, enquanto a mortalidade atingiu um máximo de 3,75%, com um valor médio de 1,05%, estando estes resultados em concordância com os observados noutros países europeus. Oito dos 21 produtores inquiridos (38,10%) afirmou ter utilizado inseticidas até dois meses antes do aparecimento dos sinais clínicos. Estes resultados salientam a importância da monitorização sistemática de *Culicoides* no contexto da vigilância epidemiológica, contribuindo para a deteção precoce e controlo de DHE.

Palavras-chave: *Culicoides*, vírus da Doença Epizootica Hemorrágica, diagnóstico molecular, RT-qPCR, Portugal

Abstract: Epizootic hemorrhagic disease (EHD), transmitted to domestic and wild ruminants by female *Culicoides* midges, is associated with high morbidity, mortality, and significant economic losses. In 2022, the disease was detected in Europe for the first time, in Italy and southern Spain. During an outbreak in Portugal (September-November 2023), *Culicoides* midges were collected in Idanha-a-Nova, Castelo Branco district, to evaluate the presence of the EHD virus in these vectors. The virus was detected in 13 of the 19 pools of biting midges analyzed (68.42%), indicating a high prevalence. Intra-herd morbidity ranged from 0.60% and 51.42%, with a mean value

of 11.34%, while mortality reached a maximum of 3.75%, with a mean value of 1.05%, which is in agreement with the frequencies observed in other European countries. Eight of the 21 inquired producers (38.10%) used insecticides until two months prior to the appearance of the first clinical signs. These results highlight the importance of systematic monitoring of *Culicoides* midges in epidemiological surveillance, contributing to the early detection and control of EHD.

Keywords: *Culicoides*, epizootic hemorrhagic disease virus, molecular diagnosis, RT-qPCR, Portugal

1. INTRODUCTION

Culicoides midges are small insects whose females are hematophagous, feeding on a wide range of vertebrate hosts, with host preference varying markedly among species (Martínez-de la Puente *et al.*, 2015). Certain species play an important role in the transmission of pathogenic agents to animals (Dähn *et al.*, 2025). Among the most relevant viral diseases transmitted by these vectors are Bluetongue, Schmallenberg disease and Epizootic Hemorrhagic Disease (EHD) (Dähn *et al.*, 2025; González *et al.*, 2025). The control of such diseases through epidemiological surveillance is of paramount importance, as it mitigates their impact on animal health by providing real-time information and contributing to the prevention of disease dissemination (Thameur *et al.*, 2021).

EHD is caused by the EHD virus (EHDV), a non-enveloped *Orbivirus* (family *Sedoreoviridae*), with a genome comprising ten segments of double-stranded RNA, that periodically results in

the death of thousands of wild and domestic ruminants worldwide (Jiménez-Cabello *et al.*, 2023). To date, seven serotypes of EHDV (EHDV-1, -2, and -4 to -8) have been identified across different regions of the world (Bibard *et al.*, 2024). In Europe, the main vectors of EHDV are *Culicoides imicola* and species belonging to the *Obsoletus* group (Bibard *et al.*, 2024; Hassine *et al.*, 2024). Serotype 8 had only been reported in Australia, in 1982, remaining restricted to this occurrence until 2021, when clinical cases were confirmed in Tunisia (Bibard *et al.*, 2024; Hassine *et al.*, 2024). Until then, EHD had never been reported in Europe; however, in the autumn of 2022, outbreaks caused by serotype 8 were detected in several European countries, including Italy and Spain, most likely originating from Tunisia (Dähn *et al.*, 2025; Bibard *et al.*, 2024).

In Portugal, a total of 79 outbreaks were confirmed between July 2023 and April 2024, resulting in the death of hundreds of cattle and causing significant economic losses in the affected regions (Spedicato *et*

al., 2024). To investigate the presence of EHDV in its vectors and to characterize the local epidemiological impact of the outbreak, entomological collections of *Culicoides* midges, together with a questionnaire survey of local producers, were carried out in Idanha-a-Nova municipality, Castelo Branco district, Portugal, between September and November 2023.

2. MATERIAL AND METHODS

2.1. Sampling site

During the EHD outbreak, between September and November 2023, a total of 19 entomological collections were performed in Idanha-a-Nova municipality, Castelo Branco district, Portugal (Figure 1).

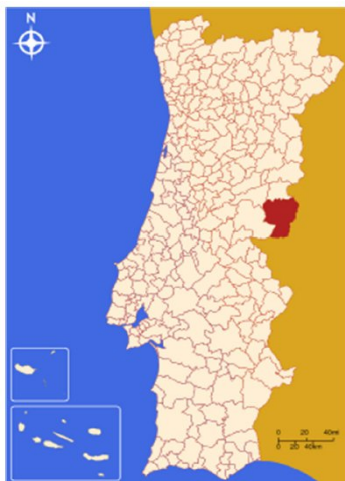


Figure 1 – Idanha-a-Nova municipality (in red).

For the collection of *Culicoides* midges, CDC miniature light traps [New Standard Miniature Black-light (UV) CDC Trap,

Model 1212, John W. Hock Company, Gainesville, Florida, USA] were installed at three different locations within one farm: i) Z1 – cattle chute; ii) Z2 – inside the barn; and iii) Z3 – fig tree, near a pond and cattle pasture areas (Figure 2). Traps were suspended 1.80 m above ground, each fitted with a collection cup containing 250 mL of 70% ethanol, a few drops of detergent and antifreeze, and 50 mL of water. Traps operated from dusk until dawn.

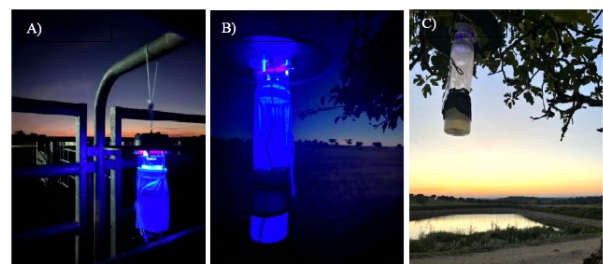


Figure 2 – CDC traps for *Culicoides* capture. A) Z1 – Cattle chute; B) Z2 – Inside the barn; C) Z3 – Fig tree, near pond and cattle pasture areas.

2.2. Morphological identification

After field collection, insects were transported to the laboratory, where they were sorted and only *Culicoides* specimens were retained for identification. Biting midges were classified by physiological state (nulliparous, blood-fed, or parous) based on abdominal coloration, and identified to genus and species level primarily based on wing patterns (Figure 3), using a taxonomical key (Ramilo, 2016). Blood-fed or parous female

Culicoides imicola midges were selected for DNA and RNA extraction.

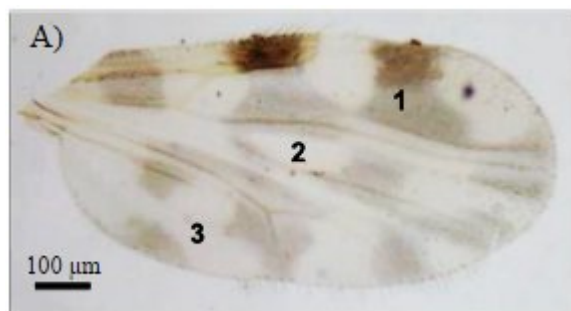


Figure 3 – *Culicoides imicola* wing pattern. 1 – Black spot with a boot-like pattern; 2 – White spot with an almond shape; 3 – White spot with an hourglass shape (Ramilo, 2016).

2.3. Molecular biology

Nineteen pools, containing between 1 and 50 insects from each capture, were processed. Specimens were subjected to mechanical lysis with 4.7-mm steel beads using a tissue homogenizer (SpeedMill PLUS, Analytik Jena) and nucleic acids (DNA and RNA) were extracted with a commercial kit (InviSorb Spin Universal Kit, Invitex Molecular, Berlin, Germany), following the manufacturer's instructions. Extracted nucleic acids were stored at -20 °C until further analysis.

To confirm the origin of the extracted genetic material, as well as to ensure the integrity of the isolated nucleic acids and the absence of DNA polymerase inhibitors, a conventional PCR targeting a fragment of

the *ITS1* gene, which is highly conserved among *Culicoides* species, was performed as described by Cêtre-Sossah *et al.* (2004). Primer sequences are described in Table 1.

The PCR amplification, using the primers PanCulF and PanCulR, was performed in a final volume of 25 μL containing 12.5 μL of 2× NZYTaQ II Green Master Mix (NZYTech®, Lisbon, Portugal), 10 pmol of each primer, 5 μL of template DNA, and sterile ultrapure water to complete the volume. For assay validation, a negative control (no DNA template) and a positive control (DNA from a known positive sample) were included in each run. The reaction was performed in a qTower thermocycler (Analytik Jena) according to the conditions described in Table 2.

PCR products were separated by electrophoresis in 1.5% (m/v) agarose gels and visualized under UV light (AlfaGene®).

Subsequently, viral RNA detection was carried out by real-time RT-PCR (RT-qPCR), targeting the *Seg-9* gene, which encodes the viral helicase, following the protocol described by Maan *et al.* (2016). The primer and probe sequences are presented in Table 3.

The amplification was performed in a final volume of 20 μ L containing 10 μ L of 2 $\times$ qScript Virus 1-Step ToughMix, Low ROX (Quantabio®), 450 nM of each primer, 150 nM of probe, 5 μ L of template RNA, and sterile ultrapure water to complete the volume. The reaction was carried out in a qTower thermocycler (Analytik Jena) according to the conditions described in Table 4. All samples were analyzed in triplicate, and samples with a mean cycle threshold (C_T) value below 40 were considered positive. In all assays, a positive control and two negative controls were included to validate the amplification and to ensure the absence of exogenous DNA contamination, respectively.

Additionally, data on morbidity and mortality rates were obtained from herds belonging to 21 producers in the same municipality, in order to assess the local impact of the outbreak. Farmers were also questioned regarding the use of insecticides or insect traps prior to the appearance of clinical cases. An informed consent was obtained from each producer.

3. RESULTS

3.1. *Culicoides* species identification

Biting midges were collected at all sampling times and across all trapping

areas. *Culicoides imicola* was the predominant species, accounting for 70.9% of the specimens collected, followed by *Culicoides punctatus* (9.4%) and specimens belonging to Obsoletus group (5.0%). The remaining 14.7% were identified as *Culicoides* spp.

3.2. Molecular biology

The amplification of a fragment of the *Culicoides* ITS1 gene was successful in all tested pools, as visualized by the presence of amplification products following agarose gel electrophoresis (Figure 4).

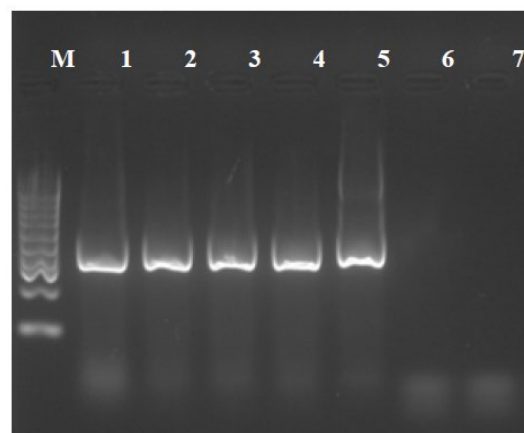


Figure 4 – Electrophoresis of *ITS1* gene fragment from *Culicoides* samples. M – Molecular weight marker (HyperLadder™ 100bp, Biolione®); 1 to 4 – PCR products of four studied samples; 5 – Positive control; 6 and 7 – Negative controls.

The amplification of the EHDV genome *Seg-9* gene by RT-qPCR was successful in 13 out of 19 pools, corresponding to 68.42% positive results (Figure 5).

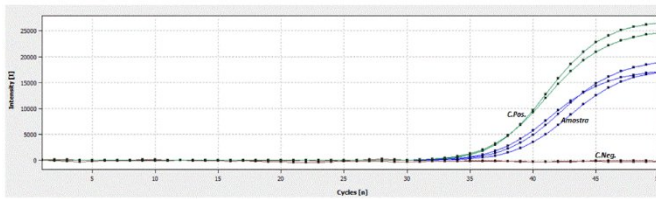


Figure 5 – Amplification of *Seg-9* gene by real-time RT-PCR from one positive sample.

Regarding the questionnaires completed by the 21 producers, the intra-flock morbidity rate ranged between 0.60% and 51.42% across all surveyed farms, with a mean of 11.34%, while the maximum mortality rate did not exceed 3.75% in any of the holdings, with a mean of 1.05%. Eight out of 21 producers (38.10%) reported the use of insecticides for pest control two months prior to the appearance of clinical signs in their animals. None of the producers reported the use of insect traps for the capture of *Culicoides* midges.

4. DISCUSSION

Epizootic Hemorrhagic Disease is transmitted by females *Culicoides* of specific species and is responsible for substantial morbidity and mortality among both wild and domestic ruminants (Jiménez-Cabello *et al.*, 2023). EHDV-8 was first reported in 1982 in Australia and, after decades without detection, re-emerged in Tunisia in 2021. Subsequently, several outbreaks were reported in Europe, including Italy and Spain in 2022, followed

by France and Portugal in 2023 (Jiménez-Cabello *et al.*, 2023; Portanti *et al.*, 2025).

The main objective of this work was to assess the prevalence of EHDV in *C. imicola* populations by detecting viral RNA. Such investigations remain scarce, as vector monitoring is typically conducted by official authorities primarily for surveillance purposes (DGAV, 2023). In our study, 68.42% of the pools tested positive for EHDV, a much higher prevalence compared to an Italian study, which reported EHDV detection in only 18.18% of *C. imicola* pools between November 2022 and February 2023 (Quaglia *et al.*, 2023). This higher prevalence in Portugal may reflect the dispersion of infected vectors from regions where the virus was already established.

To our knowledge, no other studies have assessed viral RNA detection in *Culicoides* vectors in Italy or other European countries during 2023. This lack of comparable data highlights the relevance of the present study and underscores the importance of vector-based surveillance.

Our study revealed considerable variability in morbidity rates among the surveyed herds, ranging from 0.60% to 51.42%, with a mean of 11.34%. In Spain, Zientara *et al.*, (Zientara *et al.*, 2024) reported a morbidity

rate of 6.40%, while in France, morbidity rates of 10.00% were reported (Zientara *et al.*, 2024), with another study reporting a much wider range of 1.80% to 100% among adult cattle in that country (Gondard *et al.*, 2024). In Italy, a morbidity rate of 14.09% was reported in cattle (Cresci *et al.*, 2023). The mean morbidity rate observed in our study (11.34%) was slightly higher than the <10% reported by Portuguese official data until October 2023 (DGAV, 2023), while remaining within the range reported in other European countries.

In contrast, mortality rates showed less pronounced variability in our study, ranging from 0% (in nine of the 21 herds, 42.86%) to 3.75%, with a mean of 1.05%. In Spain, a mean mortality rate of 1.70% was observed in 29 farms (Díaz-Cao *et al.*, 2025), while Zientara *et al.* (2024) reported mortality rates of 0.10% and 1.00% in cattle from Spain and France, respectively. In Italy, a mortality rate of 12.61% was reported (Cresci *et al.*, 2023). In Portugal, official data indicated mortality below 1.00% until October 2023 (DGAV, 2023). The mean mortality rate observed in our study (1.05%) was therefore slightly higher than the value reported by Portuguese official data, but lower than the mean mortality reported in Spanish farms (Díaz-Cao *et al.*, 2025) and within the range reported by Zientara *et al.* (2024).

Overall, the differences between our findings and official national estimates may be partly explained by the focused nature of our study, which targeted specific holdings and captured variability at the herd level, whereas official figures are based on aggregated national data and may therefore not fully reflect the variation between individual herds.

The use of insecticides is a common practice among Portuguese producers, with deltamethrin, cypermethrin and alphacypermethrin being the most frequently applied to reduce *Culicoides* bites during late spring and summer/early autumn. Venail *et al.* (2011) demonstrated that *C. imicola* and *Culicoides obsoletus sensu stricto* exhibit higher tolerance to deltamethrin compared to *Culicoides nubeculosus*. Page *et al.* (2014) showed that alphacypermethrin-treated meshes may enhance field efficacy by reducing biting rates. Conversely, another study reported that cypermethrin-treated nets failed to prevent the entry of *Culicoides* into enclosed spaces, and mortality rates of midges in treated and control traps did not differ significantly after 24h, suggesting no repellent or insecticidal effect against *Culicoides* populations, although other arthropods collected were susceptible (Del Río *et al.*, 2014).

The same authors attributed this to the small size of *Culicoides*, which allows them to pass through net fibers without direct contact (Del Río *et al.*, 2014).

In the present study, some producers reported using insecticides prior to the onset of clinical cases; however, EHDV infection was still observed in these holdings. The occurrence of clinical cases despite insecticide use does not necessarily indicate reduced susceptibility or resistance of *Culicoides* midges to these products, as the effectiveness of insecticide-based control may be influenced by several factors, including the timing and frequency of application, product efficacy, application methods, and the ability of the vectors to avoid treated surfaces or access untreated areas. Furthermore, no specific information on the susceptibility of the *Culicoides*

species involved to the insecticides used was available in the present study. Therefore, further studies assessing insecticide susceptibility are needed.

This study has some limitations that should be considered when interpreting the results. Entomological collections were carried out on a single farm, whereas morbidity and mortality data were obtained from a larger sample of 21 producers across the

municipality; this difference in scale should be considered when relating the observed EHDV positivity rate to the reported clinical impact. The relatively small number of pools tested ($n = 19$) also limits the precision of the positivity rate estimate. Furthermore, viral RNA detection was not accompanied by serotype confirmation or sequencing, which would have allowed direct comparison with the EHDV-8 strains circulating in neighboring countries. Interestingly, EHDV-8 RNA was subsequently detected in cattle imported from Portugal into Israel in 2023 (Golender & Hoffmann, 2024), indicating that the strain circulating during the outbreak studied here was capable of international dissemination through animal trade. Finally, the cross-sectional nature of the producer questionnaire does not allow a statistical association to be established between insecticide use and disease occurrence.

5. CONCLUSION

In conclusion, this study reinforces the need for continuous epidemiological surveillance of *Culicoides* populations, as already implemented in other countries. Such measures are critical for early detection and prevention of viral spread during peak vector activity, thereby contributing to more effective control strategies and providing farmers with

evidence-based recommendations to protect their livestock.

ACKNOWLEDGEMENTS

The authors acknowledge FMV-ULusófona for financial support through the 'Bolsas de Estágio MIMV 23-24' program.

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Tables

Table 1 - Primer sequences for amplification of the *Culicoides* spp. *ITS1* gene fragment (Cêtre-Sossah *et al.*, 2004)

Primer	Sequence (5'-3')	Amplicon size (bp)
PanCulF	GTAGGTGAACCTGCGGAAGG	316-500
PanCulR	TGCGGTCTTCATCGACCCAT	

Table 2 – PCR conditions for amplification of the *Culicoides* spp. *ITS1* gene fragment (Cêtre-Sossah *et al.*, 2004)

PCR Step	Temperature (°C)	Time (min)	Number of cycles
Initial denaturation	94	5	1
Denaturation	94	1	30
Annealing	58	1	
Extension	72	1	
Final extension	72	10	1

Table 3 – Primers and probe for detection of EHDV *Seg-9* gene (Maan *et al.*, 2016)

Primer/Probe	Sequence (5'-3')
EHDV/Seg-9/F/15-32	ATGTCAGCTGCGGTYTTG
EHDV/Seg-9/R/112-85	TCCCAATCAACTAARTGRATYTG VATCT
EHDV/Seg-9/P/69-48	6-FAM-CCTCGGTCGAACGTTGGATCAC-BHQ1

Table 4 – Real-time RT-PCR conditions for amplification of the EDHV *Seg-9* gene (Maan *et al.*, 2016)

Step	Temperature (°C)	Time	Number of cycles
Reverse transcription	55	30min	1
Initial denaturation	95	10min	1
Denaturation	95	30s	50
Annealing/Extension	60	1min	