

Culicoides species responsible for the transmission of Epizootic Haemorrhagic Disease virus (EHDV) serotype 8 in Italy

Michela Quaglia¹, Cipriano Foxi², Giuseppe Satta², Giantonella Puggioni², Roberto Bechere², Matteo De Ascentis^{1*}, Silvio Gerardo d'Alessio¹, Massimo Spedicato¹, Alessandra Leone¹, Maura Piscicella¹, Ottavio Portanti¹, Liana Teodori¹, Luigina Di Gialleonardo¹, Cesare Cammà¹, Giovanni Savini¹, Maria Goffredo¹.

¹Istituto Zooprofilattico Sperimentale dell'Abruzzo e del Molise "G. Caporale", Teramo, Italy.

²Istituto Zooprofilattico Sperimentale della Sardegna, Sassari, Italy.

*Corresponding author at: Istituto Zooprofilattico Sperimentale dell'Abruzzo e del Molise "G. Caporale", Teramo, Italy.
E-mail: m.deascentis@izs.it.

Veterinaria Italiana 2023, **59** (1), 83-88. doi: 10.12834/VetIt.3347.22208.1
Accepted: 23.08.2023 | Available on line: 30.09.2023

Keywords

Culicoides,
Epizootic Hemorrhagic
Disease virus (EHDV),
Italy,
Europe.

Summary

Epizootic haemorrhagic disease (EHD) is a viral disease transmitted by *Culicoides* biting midges that affects wild and domestic ruminants.

The causative agent, EHD virus (EHDV), belongs to the family *Sedoreoviridae*, genus *Orbivirus*. The virus has never been reported in Europe until October 2022, when the virus was for the first time detected in Sicily and Sardinia. After the first clinical cases, an intensive entomological field activity was carried out in five EHD affected farms located in Sardinia, with the aim of assessing the EHDV vector competence in European species of *Culicoides*. EHDV-8 was detected in *C. imicola*, *C. obsoletus/scoticus*, *C. newsteadi*, *C. pulicaris ss*, and *C. bysta*. The first 4 species have also been demonstrated to be able to transmit bluetongue virus (BTV). According to these results, it is likely that EHDV-8, sharing the same transmission patterns of BTV, can also spread to Europe.

Epizootic Haemorrhagic Disease (EHD) is an infectious, economic important viral disease transmitted by *Culicoides*, which affects wild and domestic ruminants.

The causative agent EHD virus (EHDV) belongs to the family *Sedoreoviridae*, genus *Orbivirus*. EHDV is closely related to the bluetongue virus (BTV). Seven serotypes (EHDV-1, EHDV-2, EHDV-4, EHDV-5, EHDV-6, EHDV-7 and EHDV-8) have been officially recognised. The World Organisation of Animal Health (WOAH) includes EHD in the list of animal disease while EU Animal Health Law, Commission Implementing Regulation 2018/1882/EU European

commission includes it in the D + E categories of the listed diseases.

First described in white-tailed deer (*Odocoileus virginianus*) in New Jersey (USA) in 1955 (Shope *et al.*, 1955), EHD is now widespread in all continents apart Antarctica. Except for the severe epidemics described in Japanese cattle because of Ibaraki virus (EHDV-2) infection, for many years EHD has been considered a serious disease of deer only. It is just recently, following the numerous outbreaks due to EHDV strains/serotypes infections observed in cattle in Northern Africa (EHDV-6-8) and Middle east (EHDV-1-7) that EHD has been included among

Please refer to the forthcoming article as: Quaglia *et al.* 2023. *Culicoides* species responsible for the transmission of Epizootic Haemorrhagic Disease virus (EHDV) serotype 8 in Italy. Vet Ital. doi: 10.12834/VetIt.3347.22208.1.

the major cattle diseases (Savini *et al.*, 2011; EFSA 2009, Sghaier *et al.*, 2022). Between October and November 2022, severe and sometimes fatal cases due to EHDV-8 infections were observed in cattle in the south-western part of Sardinia and Sicily (Lorusso *et al.*, 2023). This was the first incursion of EHDV in Italy and Europe ever recorded. Following the Italian clinical episodes, further cases of EHDV have been reported in cattle in southern Spain.

This study describes the detection of EHDV in European species of *Culicoides*, as a result of a field entomological investigation carried out during the EHDV-8 Sardinian outbreaks.

An intensive entomological field activity was performed in five EHDV affected herds: four farms (Farms 1-4) were located in Arbus and one farm (Farm 5) in Guspini municipalities (South Sardinia province) (Figure 1).



Figure 1. *Culicoides* collection sites: in red EHDV affected farms, in yellow permanent selected sites for the retrospective study.

The entomological activities have been set up immediately after the first clinical case was reported, and currently they are still in progress.

Culicoides collections were performed using UV blacklight suction traps, operating all night long from at least one hour before dusk.

As an attempt to trace back the period when EHDV-8

actually entered Italy (Sardinia), a retrospective study was also implemented, using *Culicoides* collected on a weekly basis within the entomological surveillance plan for bluetongue, and stored in ethanol 70°. For this investigation, *Culicoides* caught between August and November 2022 using the permanent traps located very close to the affected herds, in South Sardinia (SU) and in Oristano (OR) provinces, were selected (Figure 1).

All collected *Culicoides* were identified at species or complex level (Delecolle 1985, Goffredo *et al.*, 2016 and Goffredo and Meiswinkel, 2004, Sarvasova *et al.*, 2017), age graded according to Dyce (1965), and sorted in pools of maximum fifty midges. After RNA purification, each pool was tested by serogroup/serotype specific qRT-PCRs for EHDV/EHDV-8 (Sghaier *et al.*, 2022). Due to the simultaneous circulation of different serotypes of BTV in the area, collected midges were also tested by a serogroup-specific BTV real time RT-PCR assay able to detect all BTV serotypes (Hofmann *et al.*, 2008), and positive samples were tested for the presence of BTV-1, -2, -4, -8, -9 and -16 with a serotype-specific real time RT-PCR (LSI VetMAX European BTV Typing-Real-time PCR Kit - Life Technologies, Lissieu, France) and for BTV-3 with an in-house real time RT-PCR (Lorusso *et al.*, 2018).

The Minimum Infection Rate (MIR) was calculated as the number of positive pools to the total number of midges tested. The implied assumption of the MIR is that only one infected individual exists in a positive pool.

The entomological field activities collected a total of 15,339 *Culicoides* biting midges. *Culicoides obsoletus/scoticus* was the most abundant taxon, representing 56% (n= 8,621) of the total midge collected population, followed by *Culicoides imicola* (19%, n=2,898), *Culicoides pulicaris s.s.* (10%, n=1,468), *Culicoides newsteadi* (9%, n=1,368), other species (4% n=660), *Culicoides bysta* (1.5%, n=233), and *Culicoides punctatus* (0.5%, n=91) (Table I).

Overall, out of 411 tested pools (5,721 midges), 18 resulted positive to EHDV-8, consisting of 283 parous females. Of these, ten pools were composed of *Culicoides imicola* (186 midges), four of *Culicoides obsoletus/scoticus* (54 midges), two of *Culicoides pulicaris s.s.* (14 midges), one of *Culicoides newsteadi* (25 midges), and one of *Culicoides bysta* (4 midges) (Table II, Table III).

When tested for BTV/BTV-3, fifteen pools composed by *Culicoides imicola* (536 midges) resulted positive to BTV-3. Interestingly, two pools consisting of 20 and 23 *C. imicola* resulted positive to both viruses (Table II).

The details of the *Culicoides* collections found positive for EHDV-8 are shown in table III.

Table I. Relative abundance of Culicoides species collected during the Sardinian EHD outbreaks. (November 2022 – February 2023).

FARM (n. collections)	<i>C. imicola</i>	<i>C. newsteadi</i>	<i>C. punctatus</i>	<i>C. pulicaris s.s.</i>	<i>C. obsoletus/scoticus</i>	<i>C. bysta</i>	Other species	TOTAL
1 (34)	828	271	43	802	7,972	133	88	10,137
2 (25)	857	640	8	340	640	41	143	2,669
3 (3)	122	84	0	325	2	47	46	626
4 (7)	721	13	1	1	1	6	279	1,022
5 (2)	370	360	39	0	6	6	104	885
TOTAL	2,898	1,368	91	1,468	8,621	233	660	15,339

Table II. EHDV-8 and BTV-3 positive adult parous females of Culicoides collected during the Sardinian EHD outbreaks (November 2022 – February 2023).

Species	Number of EHDV-8 positive/tested pools; Number of tested midges (Minimum Infection Rate %)	Number of BTV-3 positive/tested pools; Number of tested midges (Minimum Infection Rate %)
<i>C. imicola</i>	*10/55; 912 (1.1)	*15/55; 912 (1.6)
<i>C. obsoletus/scoticus</i>	4/186; 3,542 (0.1)	0/186; 3,542
<i>C. newsteadi</i>	1/42; 365 (0.5)	0/42; 365
<i>C. pulicaris ss</i>	2/58; 572 (0.7)	0/58; 572
<i>C. bysta</i>	1/24; 78 (1.3)	0/24; 78
<i>C. punctatus</i>	0/8; 23	0/8; 23
Other species	0/38; 229	0/38; 229
TOTAL	18/411; 5,721 (0.31)	15/411; 5,721 (0.26)

* Two pools resulted positive to both viruses.

Concerning the retrospective study, 17 *Culicoides* collections were selected: ten from Villacidro (SU), and seven from Arborea (OR) (Figure 1). A total of 9,988 *Culicoides* parous were sorted in 153 pools composed of *C. imicola* (n= 8,921 midges), 16 pools of *C. newsteadi* (n=372), 6 pools of *C. punctatus* (n=40), and 35 pools composed of other species (655 midges, including *C. circumscriptus*, *C. paolae*, and others). None of the tested pools resulted positive to EHDV, but conversely 6 pools of *C. imicola* collected in Villacidro between September and November 2022, resulted positive to BTV 4.

The identification of *C. bysta* was confirmed by sequencing of partial region of the mitochondrial gene cytochrome c oxidase I (COI), using universal primers (Hebert et al., 2003). PCR products were visualized by microfluidic electrophoresis using TapeStation 4200 (Agilent Technologies), purified using GeneAll Expin™ PCR SV columns and sequenced by Eurofins Genomics (<https://eurofinsgenomics.eu/en/custom-dna-sequencing/>).

Table III. Details of EHDV-8 positive Culicoides pools collected on four affected farms (Arbus, Sardinia 2022).

Farm	Species	N. Midges	Collection date	
1	<i>C. imicola</i>	25	25/11/2022	
		18	07/12/2022	
		17	07/12/2022	
	<i>C. obsoletus/scoticus</i>	13	25/11/2022	
		25	30/11/2022	
		10	25/11/2022	
	<i>C. pulicaris</i>	4	02/12/2022	
		25	25/11/2022	
	2	<i>C. imicola</i>	11	01/12/2022
			9	07/12/2022
25			07/12/2022	
25			07/12/2022	
20			07/12/2022	
13			25/11/2022	
<i>C. obsoletus/scoticus</i>		25	25/11/2022	
3		<i>C. obsoletus/scoticus</i>	3	25/11/2022
			4	24/11/2022
4		<i>C. imicola</i>	11	25/11/2022

Sequences were then analysed using the SeqScape software and compared with GenBank database using the Basic Local Alignment Search Tool (BLAST) software. The obtained sequences showed 100% identity with *Culicoides bysta* voucher XKST01 (NCBI Accession number KY436054.1).

Our study reports the detection of EHDV-8 in pools of *C. imicola*, *C. obsoletus/scoticus* (belonging to subgenus *Avaritia*), *C. newsteadi*, *C. pulicaris ss*, and *C. bysta* (subgenus *Culicoides*) parous females caught in the EHD affected farms. Except for *C. bysta*, all these species are also known as vectors of BTV in Italy.

Culicoides imicola is known as the most important vector of BTV in countries bordering the

Mediterranean Basin (<https://ecdc.europa.eu/en/disease-vectors/surveillance-and-disease-data/biting-midge-maps>), and particularly in Sardinia. Its capability to transmit various EHDV serotypes (EHDV-2,-3,-5,-6,-7,-8) has been already demonstrated in an oral experimental infection in South Africa (Paweska et al., 2005). In our study, ten pools of *C. imicola* were also found infected with both EHDV and BTV. This is not unusual in *C. imicola* pools. Co-infection with two BTV strains/serotypes (BTV-1 and BTV-4) has been already shown in several pools collected in Sardinia (Goffredo et al., 2015).

In this survey, also *C. obsoletus/scoticus* parous females have been found positive to EHDV. Together with *Culicoides montanus*, *C. obsoletus* and *C. scoticus* are parts of the Obsoletus complex (Goffredo et al., 2016). They are cryptic species, widespread across northern and central Europe (<https://ecdc.europa.eu/en/disease-vectors/surveillance-and-disease-data/biting-midge-maps>), where *C. obsoletus/scoticus* is considered as the most important vector of BTV (Melhorn et al., 2009, Meiswinkel et al., 2008). Besides BTV, this taxon is also involved in the transmission of Schmallenberg virus (SBV) (Goffredo et al., 2013, Balenghien et al., 2014). EHDV has been detected in *C. obsoletus* pools in Turkey (Dik et al., 2012), and the presence of *C. obsoletus* has been associated to EHD in cattle and white-tailed deer in Alabama (Mullen et al., 1985). A competence study to evaluate the *C. obsoletus* and *C. scoticus* oral susceptibility to EHDV-6 also suggested the potential role of these species in transmitting EHDV (Federici et al., 2016).

Considering that the distributions of both *C. obsoletus/scoticus* and *C. imicola* cover the whole Italian and most of the European territory (Conte et al., 2007, Goffredo et al., 2016), these findings confirm that Europe, and Italy in particular, are potentially exposed to the EHDV-8 spread, as the vectors are abundant, the climate is appropriate and the hosts are available. The spreading risk is actually even higher if we consider that EHDV was also found in pools of *C. newsteadi* and *C. pulicaris* ss. These species are widely distributed in Italy and in

Central- Northern Europe (<https://ecdc.europa.eu/en/disease-vectors/surveillance-and-disease-data/biting-midge-maps>) and have already been found positive to BTV in Italy.

Unlike *C. obsoletus/scoticus* and *C. imicola*, this was the first time that EHDV was detected in *C. newsteadi* and *C. pulicaris*, as it was the first time that an *Orbivirus* was found in *C. bysta*.

Culicoides bysta is a "new" species, recently described in Slovakia (Sarvasova et al., 2017), and it has been identified for the first time in Sardinia, to update the available *Culicoides* Sardinian checklist (Foxi et al., 2011).

This species is morphologically very similar to *Culicoides pulicaris* ss, however our identification was confirmed by sequencing of partial region of the mitochondrial gene cytochrome c oxidase I (COI).

In our results, one pool consisting of four midges of *C. bysta* resulted positive to EHDV. This may suggest that this species, although not very abundant, could reach a high infection rate, possibly linked to high vector competence. However, the role of this species, implicated for the first time in the transmission of an arbovirus, needs to be better investigated.

In our retrospective study, EHDV-8 was not detected in *Culicoides* caught in the field before the outbreaks. The investigation was instead able to detect BTV-4 in *C. imicola* collected in September in Villacidro, one of the two selected farms. This finding proves that storing midges collected during the entomological surveillance program can be very useful to trace back arbovirus circulations. In the recent past, in fact, it allowed us to prove circulation of SBV in Northern Italy five months before the first detected case (Goffredo et al., 2013).

In conclusion, according to our findings, EHDV-8 seems to use the same transmission patterns of BTV. This in turn means that EHDV-8 has the potential to spread in Europe, with *C. imicola* and *C. obsoletus/scoticus* being responsible for its diffusion in the Mediterranean countries, *C. obsoletus/scoticus* acting as main vectors in Central-Northern Europe.

References

- Balenghien T., Pagès N., Goffredo M., Carpenter S., Augot D., Jacquier E., Talavera S., Monaco F., Depaquit J., Grillet C., Pujols J., Satta G., Kasbari M., Setier-Rio M.L., Izzo F., Alkan C., Delécolle J.C., Quaglia M., Charrel R., Polci A., Bréard E., Federici V., Cêtre-Sossah C. & Garros C. 2014. The emergence of Schmallenberg virus across *Culicoides* communities and ecosystems in Europe. *Prev Vet Med.* 116(4):360-9. doi: 10.1016/j.prevetmed.2014.03.007. Epub 2014 Mar 18. PMID: 24698329.
- Conte A., Goffredo M., Ippoliti C. & Meiswinkel R. 2007. Influence of biotic and abiotic factors on the distribution and abundance of *Culicoides imicola* and the *Obsoletus* Complex in Italy. *Vet Parasitol.* 150, 333-344.
- Delécolle J.C. 1985. Nouvelle contribution à l'étude systématique et iconographique des espèces du genre *Culicoides* (Diptera: Ceratopogonidae) du Nord-Est de la France. Thesis, Université Louis Pasteur de Strasbourg, UER Sciences, Vie et Terre, 238 pp.
- Dik B., Yavru S., Uslu U., Yapici O. & Esin E. 2012. Determination of *Culicoides* species (Diptera: Ceratopogonidae) as suspect vectors of epizootic haemorrhagic disease and bluetongue viruses in southern and western Anatolia by RT-PCR. *Rev Med Vet.* 163, 505-510.
- Dyce A.L. 1965. The recognition of nulliparous and parous *Culicoides* (Diptera: Ceratopogonidae) without dissection. *J Aust Entomol Soc.* 8 (1), 11-15.
- European Food Safety Authority (EFSA) & EFSA Panel on Animal Health and Welfare (AHAW). 2009. Scientific Opinion on Epizootic Hemorrhagic Disease. *EFSA Journal.* 7 (12), 1418.
- European Centre for Disease Prevention and Control (ecdc). Biting midge maps (available at <https://ecdc.europa.eu/en/disease-vectors/surveillance-and-disease-data/biting-midge-maps>. Accessed on 14 april 2023)
- Federici V., Ippoliti C., Goffredo M., Catalani M., Di Provvio A., Santilli A., Quaglia M., Mancini G., Di Nicola F., Di Gennaro A., Leone A., Teodori L., Conte A. & Savini G. 2016. Epizootic haemorrhagic disease in Italy: vector competence of indigenous *Culicoides* species and spatial multicriteria evaluation of vulnerability. *Vet Ital.* 52(3-4):271-279. doi: 10.12834/VetIt.894.4516.2. PMID: 27723036.
- Foxi C., Pinna M., Monteys V. S. I., & Del Rio G. 2011. An updated checklist of the *Culicoides* Latreille (Diptera: Ceratopogonidae) of Sardinia (Italy), and seasonality in proven and potential vectors for bluetongue virus (BTV). *Proc. Entomological Society of Washington.* 113(4), 403-416.
- Goffredo M. & Meiswinkel R. 2004. Entomological surveillance of bluetongue in Italy: methods of capture, catch analysis and identification of *Culicoides* biting midges. *Vet Ital.* 40, 260-265.
- Goffredo M., Monaco F., Capelli G., Quaglia M., Federici V., Catalani M., Montarsi F., Polci A., Pinoni C., Calistri P. & Savini G. 2013. Schmallenberg virus in Italy: a retrospective survey in *Culicoides* stored during the bluetongue Italian surveillance program. *Prev Vet Med.* 111(3-4):230-6. doi: 10.1016/j.prevetmed.2013.05.014. Epub 2013 Jun 19. PMID: 23791122.
- Goffredo M., Catalani M., Federici V., Portanti O., Marini V., Mancini G., Quaglia M., Santilli A., Teodori L. & Savini G. 2015. Vector species of *Culicoides* midges implicated in the 2012-2014 Bluetongue epidemics in Italy. *Vet Ital.* 51(2):131-8. doi: 10.12834/VetIt.771.3854.1. PMID: 26129664.
- Goffredo M., Meiswinkel R., Federici V., Di Nicola F., Mancini G., Ippoliti C., Di Lorenzo A., Quaglia M., Santilli A., Conte A. & Savini G. 2016. The '*Culicoides obsoletus* group' in Italy: relative abundance, geographic range, and role as vector for Bluetongue virus. *Vet Ital.* 52(3-4):235-241. doi: 10.12834/VetIt.35.100.1. PMID: 27723032.
- Hebert P.D., Ratnasingham S. & deWaard J.R. 2003. Barcoding animal life: cytochrome c oxidase subunit 1 divergences among closely related species. *Proc Biol Sci.* 270 Suppl 1(Suppl 1):S96-9. doi: 10.1098/rsbl.2003.0025.
- Hofmann M.A., Renzullo S., Mader M., Chaignat V., Worwa G., & Thuer B. 2008. Genetic characterization of Toggenburg orbivirus, a new bluetongue virus, from goats, Switzerland. *Emerg Infect Dis.* 14(12), 1855- 1861.
- Lorusso A., Sghaier S., Di Domenico M., Barbria M.E., Zaccaria G., Megdich A., Portanti O., Seliman I.B., Spedicato M., Pizzurro F., Carmine I., Teodori L., Mahjoub M., Mangone I., Leone A., Hammami S., Marcacci M. & Savini G. 2018. Analysis of bluetongue serotype 3 spread in Tunisia and discovery of a novel strain related to the bluetongue virus isolated from a commercial sheep pox vaccine. *Infect Genet Evol.* 59:63-71. doi: 10.1016/j.meegid.2018.01.025. Epub 2018 Jan 31. PMID: 29386141.
- Lorusso A., Cappai S., Loi F., Pinna L., Ruiu A., Puggioni G., Guercio A., Purpari G., Vicari D., Sghaier S., Zientara S., Spedicato M., Hammami S., Ben Hassine T., Portanti O., Breard E., Sailleu C., Ancora M., Di Sabatino D., Morelli D., Calistri P. & Savini G. 2022.

- Epizootic Hemorrhagic Disease Virus Serotype 8, Italy, *Emerg Infect Dis*. 2023 May;29(5):1063-1065. doi: 10.3201/eid2905.221773. PMID: 37081599; PMCID: PMC10124640.
- Meiswinkel R., Baldet T., De Deken R., Takken W., Delécolle J.C. & Mellor P.S. 2008. The 2006 outbreak of bluetongue in northern Europe - the entomological perspective. *Prev Vet Med*, 87 (1), 55-63.
- Mehlhorn H., Walldorf V., Klimpel S., Schaub G., Kiel E., Focke R., Liebisch G., Liebisch A., Werner D., Bauer C., Clausen H., Bauer B., Geier M., Hörbrand T., Bätza H.J., Conraths F.J., Hoffmann B. & Beer M. 2009. Bluetongue disease in Germany (2007-2008): monitoring of entomological aspects. *Parasitol Res.*, 105(2):313-9. doi: 10.1007/s00436-009-1416-y. Epub 2009 Mar 26. PMID: 19322587.
- Mullen G.R., Hayes M.E. & Nusbaum K.E. 1985. Potential vectors of bluetongue and epizootic hemorrhagic disease viruses of cattle and white-tailed deer in Alabama. *Progr Clin Biol Res*, 178, 201-206
- Paweska J.T., Venter G.J. & Hamblin C. 2005. A comparison of the susceptibility of *Culicoides imicola* and *C. bolitinos* to oral infection with eight serotypes of epizootic haemorrhagic disease virus. *Med Vet Entomol*, 19 (2), 200-207.
- Sarvašová A., Kočíšová A., Candolfi E. & Mathieu B. 2017. Description of *Culicoides (Culicoides) bysta* n. sp., a new member of the Pulicaris group (Diptera: Ceratopogonidae) from Slovakia. *Parasit Vectors*. 10(1):279. doi: 10.1186/s13071-017-2195-4. PMID: 28578677; PMCID: PMC5457568.
- Savini G., Afonso A., Mellor P., Aradaib I., Yadin H., Sanaa M., Wilson W., Monaco F. & Domingo M. 2011. Epizootic haemorrhagic disease. *Res Vet Sci*, 91, 1-17.
- Sghaier S., Sailleau C., Marcacci M., Thabet S., Curini V., Ben Hassine T., Teodori L., Portanti O., Hammami S., Jurisic L., Spedicato M., Postic L., Gazani I., Ben Osman R., Zientara S., Bréard E., Calistri P., Richt J.A., Holmes E.C., Savini G., Di Giallonardo F. & Lorusso A. 2022. Epizootic Haemorrhagic Disease Virus Serotype 8 in Tunisia, 2021. *Viruses*. 15(1):16. doi: 10.3390/v15010016. PMID: 36680057; PMCID: PMC9866946.
- Shope R.E., MacNamara L.G. & Mangold R., 1955. Epizootic Haemorrhagic Disease of deer. *New Jersey Outdoors* (November) 16, 21.