

Epizootic haemorrhagic disease in Italy: vector competence of indigenous *Culicoides* species and spatial multicriteria evaluation of vulnerability

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Vector competence.

Summary

Epizootic haemorrhagic disease (EHD) is an infectious non-contagious viral disease transmitted by *Culicoides*, which affects wild and domestic ruminants. The disease has never been reported in Europe, however recently outbreaks of EHD occurred in the Mediterranean Basin. Consequently, the risk that Epizootic haemorrhagic disease virus (EHDV) might spread in Italy cannot be ignored. The aim of this study was to evaluate the risk of EHDV transmission in Italy, in case of introduction, through indigenous potential vectors. In Italy, the most spread and abundant *Culicoides* species associated to livestock are *Culicoides imicola* and the members of the *Obsoletus* complex. *Culicoides imicola* is a competent vector of EHDV, whereas the vector status of the *Obsoletus* complex has not been assessed yet. Thus, its oral susceptibility to EHDV was here preliminary evaluated. To evaluate the risk of EHDV transmission a geographical information system-based Multi-Criteria Evaluation approach was adopted. Distribution of vector species and host density were used as predictors of potential suitable areas for EHDV transmission, in case of introduction in Italy. This study demonstrates that the whole peninsula is suitable for the disease, given the distribution and abundance of hosts and the competence of possible indigenous vectors.

Malattia emorragica epizootica del cervo in Italia: competenza vettoriale delle specie di *Culicoides* indigene e valutazione del rischio di diffusione della malattia

Parole chiave

Competenza vettoriale,
Culicoides obsoletus,
Culicoides scoticus,
Italia,
Malattia emorragica
epizootica del cervo,
Sistema informativo
geografico,
Valutazione
multifattoriale.

Riassunto

La Malattia emorragica epizootica del cervo (EHD) è una malattia virale non contagiosa che colpisce i ruminanti domestici e selvatici, trasmessa da ditteri appartenenti al genere *Culicoides*. Il virus dell'EHD (EHDV) non è mai stato segnalato finora in Europa, tuttavia, recentemente, alcuni focolai si sono verificati nel bacino del Mediterraneo. Pertanto, a seguito di una eventuale introduzione, non si può trascurare il rischio di diffusione dell'EHDV nel territorio italiano. Lo scopo di questo studio è quello di valutare il rischio di diffusione dell'EHDV in Italia, in caso di introduzione, attraverso i potenziali vettori autoctoni. In Italia, le specie di *Culicoides* associate al bestiame, più abbondanti e diffuse, sono *Culicoides imicola* e l'*Obsoletus* complex. *Culicoides imicola* è un vettore ben noto dell'EHDV, mentre il ruolo svolto dall'*Obsoletus* complex non è ancora noto. In questo lavoro viene valutata la competenza vettoriale di entrambe le specie, *C. obsoletus* e *C. scoticus*, appartenenti all'*Obsoletus* complex, attraverso l'infezione sperimentale tramite pasto di sangue infetto, in condizioni di laboratorio. Per valutare il rischio di diffusione dell'EHDV è stato adottato un approccio GIS basato su metodi di analisi multicriterio. Per creare le mappe di vulnerabilità, sono state utilizzate le distribuzioni dei vettori e le densità della popolazione bovina in Italia. Lo studio individua le aree potenzialmente favorevoli alla diffusione dell'EHDV, e mostra come il virus si potrebbe diffondere in tutto il territorio italiano, in base alla distribuzione e abbondanza degli ospiti ed alla competenza vettoriale dei possibili vettori autoctoni.

Introduction

Epizootic haemorrhagic disease (EHD) is an infectious non-contagious viral disease transmitted by *Culicoides*, which affects wild and domestic ruminants. Epizootic haemorrhagic disease virus (EHDV) belongs to the genus *Orbivirus*, family *Reoviridae*. At least 7 serotypes have been recognised and reported in North America, Australia, Asia, and Africa (Savini et al. 2011). Susceptible hosts are wild cervids, in particular white-tailed deer (*Odocoileus virginianus*) of North America (Gaydos et al. 2004, Murphy et al. 2006). Severe clinical signs have also been observed in cattle infected by EHDV serotypes 2, 6, and 7, whereas the role of sheep and goats as hosts is uncertain (Savini et al. 2011). In cattle, mortality is generally low while morbidity varies from 1% to 18% (EFSA 2009); the viraemia can persist for up to 50 days after infection (Gibbs and Lawman 1977), suggesting that cattle acts as a reservoir for virus. The economic repercussions are due to deaths, production losses, movement restrictions and ban of trading livestock in infected areas (EFSA 2009).

Epizootic haemorrhagic disease virus is widespread worldwide, affecting areas of North and Central Americas, of South and Central Africa, of Middle East, of Australia, and areas of Southeast Asia (Savini et al. 2011). The disease has never been reported in Europe, however recently (2006–2007) outbreaks of EHD, caused by serotypes 6 and 7, occurred in countries facing the Mediterranean Basin (Morocco, Algeria, Tunisia, Israel, Jordan, and Turkey) (OIE 2006 a, b, c, d, Temizel et al. 2009, Yadin et al. 2008). In view of these recent outbreaks, the risk that EHDV might spread in Italy cannot be ignored.

Several incursions of the virus outside its endemic region could be traced to subclinically infected animals or infected vectors, thus the risk is linked to animal movement and possibly to vector transportation by wind. Consequently, the spreading of the disease following introduction would depend on the interactions among animal hosts, vectors and virus, including the availability of susceptible hosts and the vectorial capacity of the local indigenous vector populations. The vector capacity of an insect species to an arbovirus, in a given area where susceptible hosts are available, depends essentially on its vector competence and abundance.

In Italy, the most spread and abundant *Culicoides* species associated to livestock are *Culicoides imicola* and the members of the *Obsoletus* complex (Goffredo et al. 2004). The *Obsoletus* complex is a group of cryptic species, including *Culicoides obsoletus*, *Culicoides scoticus* and *Culicoides montanus*, undistinguishable morphologically and identified only by molecular tools (Gomulski et al. 2005). *Culicoides imicola* is a competent vector of

EHDV (Aradaib et al. 1999, Paweska et al. 2005), whereas the vector status of the species belonging to the *Obsoletus* complex has not been assessed yet. For this reason, their oral susceptibility to EHDV under laboratory conditions has been preliminary evaluated in this study.

Disease outbreaks may occur in areas where populations of ruminants and potential vectors are both present: Italy is vulnerable to EHDV introduction because of the combination of generally favourable eco-climatic conditions, of the presence of bovines, as well as of potential vectors. Risk-based disease surveillance and control systems can be designed for an early detection of disease, and they can benefit from the production of risk maps (Stevens et al. 2013).

When data are scarce or disease has not been yet detected as the case of EHD in Italy, a way to evaluate the disease risk of introduction is based on the knowledge of the disease epidemiology and the risk factors proved in other places, properly transferred. Multi-Criteria Evaluation (MCE) approach, or Multi Criteria Decision Analysis (MCDA), is a knowledge-driven modelling method, which uses current knowledge regarding the factors associated with disease occurrence as model inputs in order to identify areas suitable for disease occurrence (Stevens et al. 2013). A geographical information system (GIS)-based multi-criteria approach has been recently used in animal disease (Stevens et al. 2013) and emerging viral zoonosis (Tran et al. 2013, Sanchez-Vizcaino et al. 2013, Clements et al. 2006) to model the habitat suitability for occurrence of highly pathogenic avian influenza virus in domestic poultry in Asia, as well as to map the areas at risk for Rift Valley Fever vector-borne transmission in Italy, in Spain, and in Africa continent, respectively.

This study had the goals of: i) investigate, for the first time, the vector competence, in laboratory environment, of the cryptic species of the *Obsoletus* complex (*C. obsoletus* and *C. scoticus*) for EHDV-6, and ii) evaluate the vulnerability of Italy to EHDV transmission, in case of introduction, through indigenous potential vectors, by implementing a GIS-based Multi criteria Evaluation approach.

Materials and methods

Oral infection

Experimental infections were performed through artificial oral infection of wild-caught *Culicoides* collected at a horse stable in Teramo (Abruzzo, Italy) in June, July, and September 2014. Defibrinated horse blood infected with EHDV serotype 6 was offered to wild caught midges through cotton wool

pledgets in accordance to the method described by Venter and colleagues (Venter *et al.* 2005). The titre of virus in blood meals ranged between $10^{3.86}$ TCID₅₀/ml and $10^{5.23}$ TCID₅₀/ml.

Post-feeding *Culicoides* were immobilized on dry ice and engorged females, belonging to the *Obsoletus* complex, were sorted out on a refrigerate chill-table. Species identification was performed according to Delécolle, Campbell and Pelham-Clinton, Goffredo and Meiswinkel (Delécolle 1985, Campbell and Pelham-Clinton 1960, Goffredo and Meiswinkel 2004). Some of midges were analysed immediately after feeding (0 days post infection, dpi). The other fed midges were incubated for 10 days at 25°C and then killed and stored at -80°C until tested individually (as whole insects) for EHDV by real time polymerase chain reaction (RT-PCR) (Clavijo *et al.* 2010). Samples with a threshold cycle (Ct) value less than 45 were classified as positive and those with Ct > 45 as negative.

Finally, positive midges were molecularly identified at species level by a multiplex PCR (Gomulski *et al.* 2005) and tested by virus isolation (OIE 2014).

MCE approach

The key steps of Multi-Criteria Evaluation approach are: identifying the 'risk' factors (vector and host layers); determining their relative importance (weights); combining them into a cartographic map of areas suitable to virus establishment (vulnerability maps); validation of the results. The hypothesis is that, if in a location the risk factors co-occur, that area is more vulnerable to the disease event.

Vector and host data

The data used in this study were:

- abundance of *C. imicola* in 1,205 geo-referenced sites in Italy,
- abundance of *Obsoletus* complex in 860 geo-referenced sites in Italy,
- cattle density [number of animals / km² reported in the Italian National Data Base (NDB) for Livestock Registration at 31.08.2014] per municipality.

Entomological data derived from the entomological activities performed in Italy from 2000 to 2013, within the Bluetongue Surveillance National Plan. The maximum number of specimens reported per each site was considered representative of the abundance of the species/complex in the location. To avoid false negative values, only collections repeated at least 3 and 2 times in the same location, for *C. imicola* and *Obsoletus* complex respectively, or with a maximum number of captured specimens

greater than 1,000 for *C. imicola* and 30 for *Obsoletus* complex, were considered.

The ordinary Kriging method was used to derive data in unsampled locations, optimizing models in Geostatistical Analyst Module in ESRI ArcMap® 10 software.

The mean number of specimens per municipality was extracted from Kriging maps through Zonal Statistics tool in ESRI ArcMap® 10 software.

Also data on cattle densities were calculated for each Italian municipality. Bovine data derived from Animal Identification and Registration System National Database (NDB - National Livestock Database), maintained and managed at Istituto Zooprofilattico Sperimentale dell'Abruzzo e del Molise (IZSAM) on behalf of the Italian Ministry of Health.

To assign a common risk scale 0-1 to each risk factor, fuzzy membership function (Malczewski 1999) has been used in IDRISI Taiga software. We assumed a linear, monotonically increasing relationship between each data layer (logarithmic scale for vector abundances) and vulnerability, i.e. we assumed that the higher the abundances, the higher the capacity, the higher is the vulnerability to disease event.

To combine the data, the relative importance of each factor was determined by assuming that both the presence of cattle and vectors is required for the disease occurrence. Weights for each layer have been calculated in IDRISI Taiga software and derived from a pairwise comparison matrix of the analytical hierarchy process (Malczewski 1999). The importance (i.e. weight) of each factor was compared to the others in contributing to the final solution and a value was assigned to each factor rated on a 9-point scale. The values of the scale range from 1/9 extremely less important, 1/7, 1/5, 1/3, to 1 equally important, 3, 5, 7 to 9 extremely more important.

A matrix of n x n elements (factors) was built and the principal eigenvector of this matrix was calculated to derive the best-fit set of weights.

Two set of weights have been derived, for the 2 scenarios:

- scenario 1: *C. imicola* is a 'more competent' vector for EHDV than the *Obsoletus* complex;
- scenario 2: the 2 taxa act with the same competence for EHDV transmission.

Results

Oral infection

Epizootic haemorrhagic disease virus serotype 6 was recovered by real time RT-PCR from 10 midges out of 40 (25%) of the *Obsoletus* complex tested

Table I. Vector competence study: virus detection by real time RT-PCR in midges of the *Obsoletus* complex at 0 and 10 days post infection (dpi).

Species	Incubation period (dpi)	N. positive	N. tested	Infection rate (%)
Obsoletus complex	0	10	40	25
	10	6	962	0.6

immediately after feeding, and from 6 midges out of 962 (0.6%) incubated for 10 days (Table I). Positive midges included both *C. obsoletus* and *C. scoticus* species of the complex (Table II). The virus isolation, attempted on real time RT-PCR positive midges, was unsuccessful.

Vector and host maps

The maps showing the rescaled suitability values for *C. imicola* (Figure 1), *Obsoletus* complex (Figure 2) and cattle densities in Italian municipalities (Figure 3) have a continuous scale in [0-1] interval, ranging from low to high vulnerability locations. The trap locations are also shown on the vector maps as points.

The maps highlight spatially heterogeneous patterns of vectors and host distributions on Italian territory, the distribution of the 2 vectors being almost complementary.

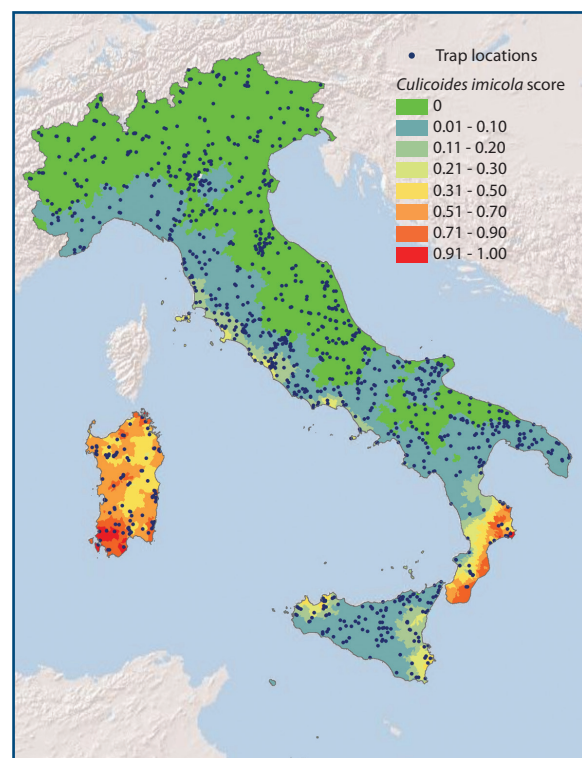
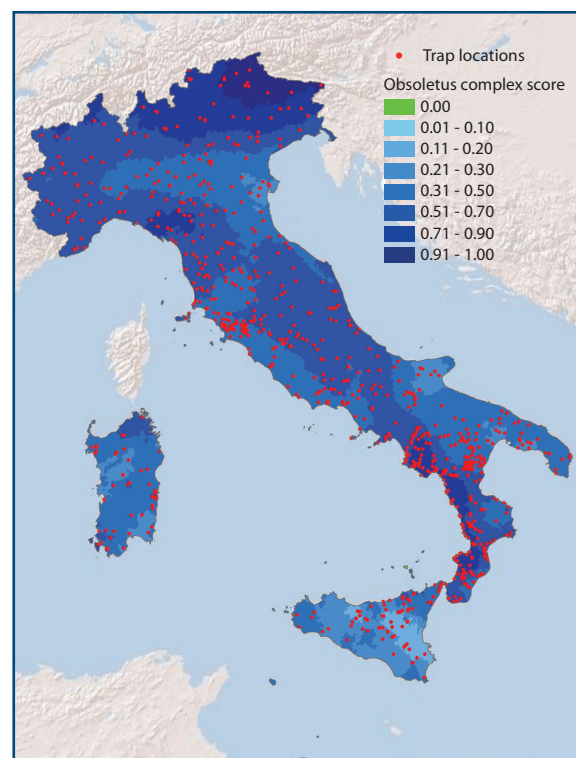
Table II. Vector competence study: species identification of the Epizootic haemorrhagic disease virus positive midges and threshold cycle (Ct) values.

Species	Incubation period (dpi)	N. midges	Mean Ct value	Min Ct value	Max Ct value
<i>C. obsoletus</i>	0	3	34.7	25	40
	10	1	42.0	42	42
<i>C. scoticus</i>	0	7	39.4	36	43
	10	5	41.8	38	43
Total	0	10	38.0	25	43
	10	6	41.8	38	43

Cattle density has a scattered distribution across the peninsula, with higher concentrations in the North (Piedmont, Lombardy, Veneto, part of Emilia Romagna regions) and some areas of Apulia and Sicily regions.

MCE results

Table III shows the weights of the models: cattle density is the highest weighted vulnerability factor in both scenarios, while *C. imicola* and *Obsoletus* complex receive lower weighting (same weight in the second scenario).

**Figure 1.** Distribution and abundance of *Culicoides imicola* in Italy: the values range in a continuous scale from least (0) to most (1) abundance per municipality. Source: Entomological Surveillance Plan for Bluetongue, 2000-2013.**Figure 2.** Distribution and abundance of *Obsoletus* complex in Italy: the values range in a continuous scale from least (0) to most (1) abundance per municipality. Source: Entomological Surveillance Plan for Bluetongue, 2000-2013.

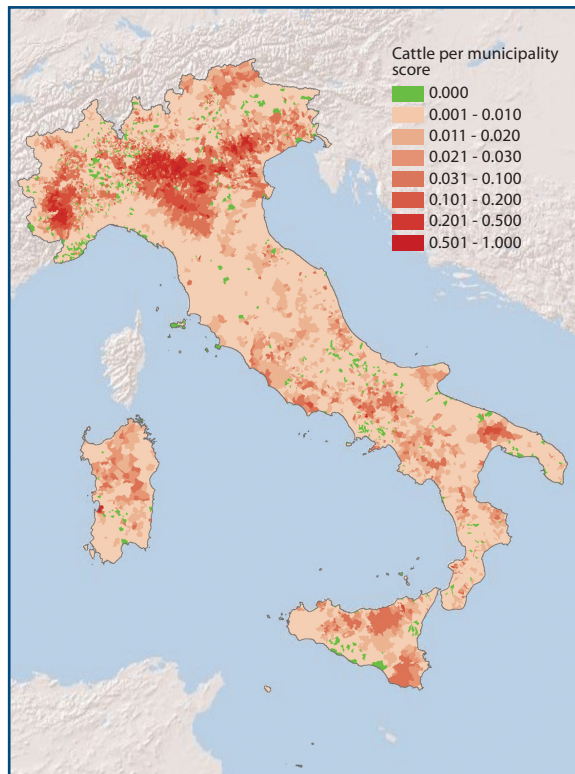


Figure 3. Distribution of cattle densities in Italy (number of animals/sqkm): the values range in a continuous scale from least (0) to most (1) abundance per municipality. Source: National Livestock Database, 31.08.2014.

Table III. Pairwise comparison matrix of the analytical hierarchy process (AHP) for risk factors associated with Epizootic haemorrhagic disease and weights of the models, considering host density as the major risk factor, *Culicoides imicola* as the second factor and the *Obsoletus* complex as third and 'less important' factor (scenario 1). In bracket are reported the values for scenario 2 (*Culicoides imicola* and *Obsoletus* complex acting with the same capability).

	Cattle density	<i>C. imicola</i> abundance	<i>Obsoletus</i> complex abundance	Weight
Cattle density	1 [1]			0.5396 [0.5]
<i>C. imicola</i> abundance	½ [½]	1 [1]		0.2970 [0.25]
<i>Obsoletus</i> complex abundance	1/3 [½]	½ [½]	1 [1]	0.1634 [0.25]

Figure 4 depicts the areas potentially suitable for virus establishment in Italy, considering *C. imicola* 'more competent' vector for EHD transmission than the *Obsoletus* complex (scenario 1). Scenario 2 vulnerability map, i.e. considering *Obsoletus* complex 'as competent' as *C. imicola* vector for EHD, is shown in Figure 5.

The maps are displayed on a continuous scale from least to most suitability represented by green-red colour scale. Dark-red areas are considered at

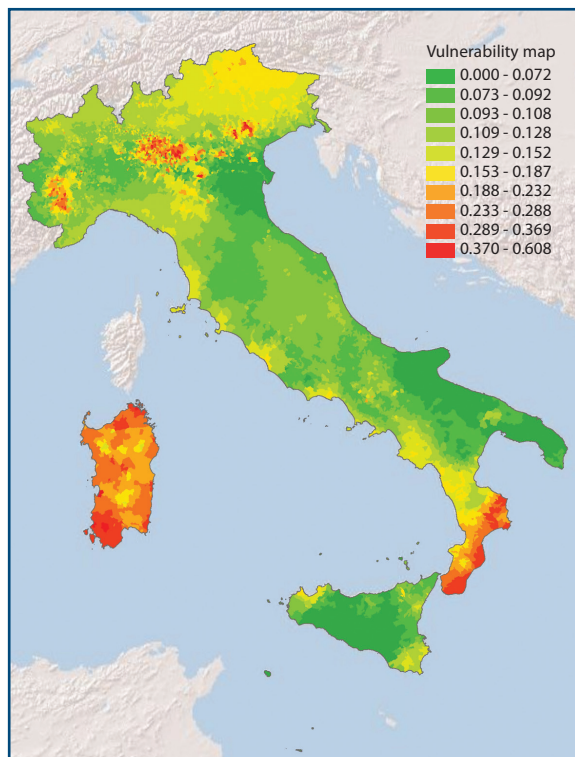


Figure 4. Vulnerability map for Epizootic haemorrhagic disease virus transmission among cattle in Italy, with *Culicoides imicola* acting as the principal vector and the *Obsoletus* complex playing a minor role (scenario 1).

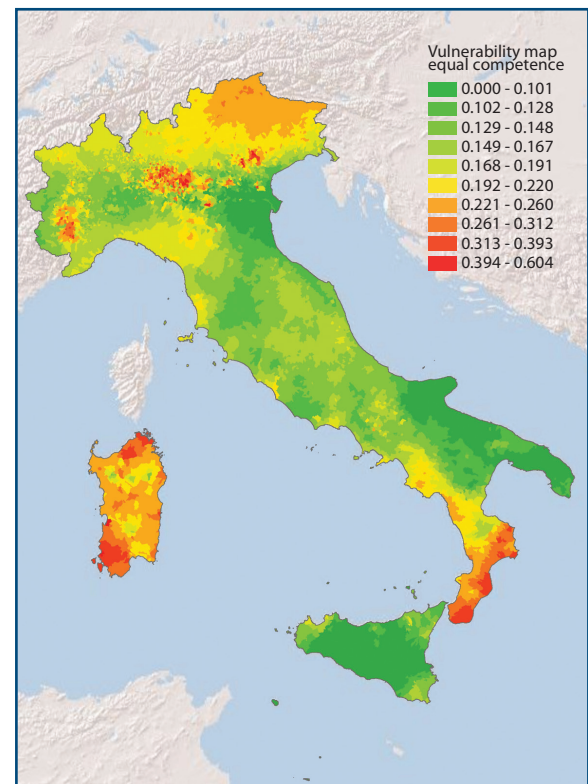


Figure 5. Vulnerability map for Epizootic haemorrhagic disease virus transmission among cattle in Italy, with *Culicoides imicola* and the *Obsoletus* complex acting as vectors with the same capability (scenario 2).

higher vulnerability for EHDV spread, yellow areas moderately vulnerability, while light and dark-green indicates areas at low and very low vulnerability, respectively.

Discussion

This study investigated, for the first time, the vector competence for EHDV of the *Obsoletus* complex (*C. obsoletus* and *C. scoticus*), under laboratory conditions, and mapped the areas potentially suitable for disease spread in Italy.

The oral infection, reported in this study, showed that the *Obsoletus* complex was susceptible to EHDV, at least serotype 6, since the virus was detected from *C. obsoletus* and *C. scoticus* after the extrinsic incubation period.

The EHDV serotype 6 has been chosen according to the risk of its introduction in Italy, considering its recent incursions in the Mediterranean countries.

To assess the vector competence of a *Culicoides* species for a virus, 4 criteria should be satisfied (WHO 1967):

1. association of species to the disease in the field;
2. recovery of virus from wild-caught females whose abdomens are free from fresh blood;
3. demonstration of its infection after feeding on viraemic host or artificial substitute;
4. demonstration of its ability to transmit the infection biologically.

Culicoides imicola is already considered a vector of EHDV, since at least the first 3 criteria are satisfied (Aradaib *et al.* 1999, Paweska *et al.* 2005). Conversely, the *Obsoletus* complex should be considered a suspected vector, since the literature reports only some associations of *C. obsoletus* with cattle and white-tailed deer affected with EHDV in Alabama (Mullen *et al.* 1985) and the finding of EHDV in *C. obsoletus* pools in Turkey (Dik *et al.* 2012). In this study, the recovery of EHDV by real time RT-PCR from both *C. obsoletus* and *C. scoticus* implies a feasible infection of these species after feeding on a viraemic artificial blood meal.

However, the high Ct values detected at 10 dpi for both species (Table II) and the unsuccessful isolation of virus, do not support the evidence of virus replication in the salivary glands (Veronesi *et al.* 2013). Nevertheless, the minimum Ct value of *C. scoticus* at 10 dpi (38) was lower than the Mean Ct value at 0 dpi (39.4), suggesting the possibility of virus replication.

Several Southafrican *Culicoides* species, including *C. imicola*, have been fed with various EHDV serotypes. The virus recovery rate for *C. imicola*, at 10

days after oral infection, ranged from 0.4% for EHDV-2 to 14.4% for EHDV-7, in particular it was 3.4% for serotype 6 (Paweska *et al.* 2005). The *Obsoletus* complex, in this study, showed a lower virus recovery rate (0.6%; Table I), compared to *C. imicola* (Paweska *et al.* 2005).

However, the methods used are different for virus detection (virus isolation vs RT-PCR) and for feeding (1 day chicken membrane vs cotton wool pads). In particular, the cotton wool pads method could have reduced infection rates for the smaller volume of blood meal taken up (Venter *et al.* 2005). In addition, the infected meals in this study had a lower virus titre when compared to *C. imicola* fed with EHDV-6 (Paweska *et al.* 2005). This can justify the lower recovery rate in midges at 0 dpi (25%; Table I), since it is correlated to the virus titre of the blood meal (Paweska *et al.* 2005). Yet, this may not necessarily equate to low vectorial capacity. In fact the possible low susceptibility of *Obsoletus* complex to infection with EHDV could be compensated by its abundance and wide distribution (Figure 2).

Even if further investigations are required to understand better the vectorial role of the *Obsoletus* complex, this study provides, for the first time, a preliminary evidence that the *Obsoletus* complex could be considered competent vector of EHDV. In particular, since both the cryptic species *C. obsoletus* and *C. scoticus* were found positive at 10 dpi, the whole complex was considered a risk factor in the GIS-based Multi-Criteria Evaluation approach.

Culicoides imicola is geographically complementary to *Obsoletus* complex in Italy (Conte *et al.* 2007), thus the 2 taxa together cover the whole Italian peninsula.

As it is not possible to establish if the species of the *Obsoletus* complex would play a similar/minor/major role compared to *C. imicola* in the field, 2 scenarios were proposed: *Obsoletus* complex has a minor role in the transmission of the virus (scenario 1) or *C. imicola* and *Obsoletus* complex act as vector with the same competence (scenario 2).

The results show that, if EHDV appeared in Italy, it would be able to spread from North to South according to the abundance of vectors. *Obsoletus* complex and *C. imicola* distributions together cover the entire Italian territory (Conte *et al.* 2007) and, for this reason, the whole peninsula could be considered vulnerable to the virus.

Nevertheless, the 2 final maps indicate a greater vulnerability level in areas with a higher livestock and vectors densities. In particular, Sardinia Island and most of Calabria region are more vulnerable in both scenarios (due mostly to high abundance of *C. imicola*), whereas the Northern areas of the country are at higher vulnerability (Piedmont,

Lombardy, Veneto) because of the higher cattle population density. Furthermore, the North-East of Italy increase his vulnerability in the second scenario, with the Obsoletus complex considered as competent as *C. imicola*, since this complex is very abundant in this area. The zones in which hosts are not so abundant and vectors are few or absent (part of Emilia Romagna, regions of Central Italy, Apulia, and Sicily regions) resulted less vulnerable in both scenarios.

The possibility that the Obsoletus Complex may be more competent than *C. imicola*, as well as the possibility that other European species may be competent vectors for EHDV, could not be excluded. *Culicoides punctatus*, for instance, can be considered a potential vector (Yanase *et al.* 2005). Therefore the risk reported in this study could be underestimated.

The seasonal abundance of vectors has an important impact in the efficiency of virus transmission. *Culicoides imicola* has his favourable activity period in late Summer, while the Obsoletus complex has 1 or 2 seasonal peaks, in late Spring and, sometimes, in Autumn. In view of this, the virus transmission would arise more rapidly if the introduction occurs during the vectors' seasonal peaks.

Deers are susceptible to EHDV and can be viraemic for 56 days (Quist *et al.* 1997), thus their involvement in virus transmission cannot be excluded. In 2010, approximately 543,000 cervids (*Cervus elaphus*, *Capreolus capreolus*, *Dama dama*) were reported, with a predominance of *Capreolus capreolus*, followed by *Cervus elaphus* and *Dama dama* (Raganella Pelliccioni *et al.* 2013). They have an inhomogeneous distribution, in particular the populations in the Central and Southern Italy are reduced and fragmented. *Cervus elaphus* and *C. capreolus* have a more continuity in the Alps, whereas *D. dama* is almost absent.

In this study only cattle are considered as susceptible hosts because detailed data on Italian population of cervids is lacking. Although in Italy their consistency is only about 10% of cattle population cervid role should be better investigated in further development.

The GIS-based Multi Criteria Evaluation approach offers a valuable framework and flexible tool for mapping pathogen at risk areas in a free region, providing policy-makers with suitable information on where and how to target surveillance and preventive actions (Tran *et al.* 2013).

This approach allows for integrating the available knowledge about potential vectors and host distribution in order to provide a risk maps for EHD transmission in Italy. This is a knowledge-driven model (Stevens *et al.* 2013), particularly useful in situations like this, where data are not available (because the disease is not present) or they are very scanty. Even if the validation of the model is not possible, MCE allows for identifying the key parameters that have a significant impact on the resulting maps, and permit to identify the main knowledge gaps (Tran *et al.* 2013).

It is worth noticing that the huge database derived from the surveillance activities, contains detailed entomological information on Italy at remarkable spatial, temporal and species resolution. Moreover, at the Animal Identification and Registration System National Database (NDB - National Livestock Database), individual register for bovine animals and every movement (passport), birth or death are reported, together with information concerning the structures holding animals and any animal movement. The valuable quality and quantity of data input, guarantee the goodness of the output of data model, even in the case of absence of validation procedures.

In conclusion, this first attempt to map the vulnerability of Italian peninsula to EHDV spread, in case of disease introduction, leads to consider the whole peninsula suitable for the disease, due to the host distribution and to the competence, distribution and abundance of *C. imicola* and Obsoletus complex. The model would benefit from adding other risk factors or considering the temporal component, and the animal movements, to define the spatio-temporal areas more suitable to virus spread.

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