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Detection of the new emerging rabbit haemorrhagic disease type 2 virus (RHDV2) in Sicily from rabbit (*Oryctolagus cuniculus*) and Italian hare (*Lepus corsicanus*)



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ABSTRACT

Rabbit haemorrhagic disease virus (RHDV), a member of the genus *Lagovirus*, causes rabbit haemorrhagic disease (RHD), a fatal hepatitis of rabbits, not previously reported in hares. Recently, a new RHDV-related virus emerged, called RHDV2. This lagovirus can cause RHD in rabbits and disease and mortality in *Lepus capensis* (Cape hare). Here we describe a case of RHDV2 infection in another hare species, *Lepus corsicanus*, during a concurrent RHD outbreak in a group of wild rabbits. The same RHDV2 strain infected rabbits and a hare, also causing a RHD-like syndrome in the latter. Our findings confirmed the capability of RHDV2 to infect hosts other than rabbits and improve the knowledge about the epidemiology and the host range of this new lagovirus.

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Rabbit haemorrhagic disease virus (RHDV), genus *Lagovirus*, is well known for causing rabbit haemorrhagic disease (RHD), which only affects *Oryctolagus cuniculus* (domestic and wild rabbits). The disease is highly contagious and usually fatal, with mortality range of 70–100% (OIE, 2010). The gross lesions include hyperaemia, congestion and diffuse haemorrhages, liver degeneration, necrosis and splenomegaly (Xu and Chen, 1989). A similar disease, European brown hare syndrome (EBHS), mainly occurs in European brown hares (*Lepus europeus*), but has also been reported in *Lepus timidus* and *Lepus corsicanus*. EBHS is characterized by mild nervous signs, severe necrotic hepatitis and circulatory dysfunction (Gavier-Widén and Mörner, 1991). The causative agent of EBHS is the European brown hare syndrome virus (EBHSV), which also belongs to the *Lagovirus* genus (Capucci et al., 1991; Wirblich et al., 1994). Although highly related, RHDV and EBHSV are classified as two viral species, phylogenetically distinct, which have a restricted host specificity both naturally and experimentally (Bergin et al., 2009; Lavazza et al., 1996). In 2010, a variant of RHDV was detected in France during RHD outbreaks in vaccinated rabbits. Further studies on the variant

indicated that it could not have evolved from RHDV but represented a new virus originating from an unknown viral species, leading Le Gall-Reculé et al. (2013) to propose the variant as a new lagovirus species, named RHDV2. This conclusion has been supported by the recent finding of Puggioni et al. (2013), who reported several cases of EBHSV-like disease in *Lepus capensis mediterranea* (Cape hares) caused by RHDV2. The latter has been also reported to be rapidly spreading in Europe, and has caused significant losses in farmed and wild rabbits in France (Le Gall-Reculé et al., 2013), Italy (Puggioni et al., 2013), Portugal (Abrantes et al., 2013), Spain (Dalton et al., 2014), Germany and the United Kingdom (Westcott et al., 2014).

In this paper we describe a single case of RHDV2 infection in an Italian hare (*Lepus corsicanus*), which occurred during a RHD outbreak in rabbits. During December 2012, RHD was diagnosed in wild rabbits living in an area of Syracuse province, Sicily, Italy (37°01'42" N, 15°15'24" E). The rabbitry consisted of 40 individuals, which were kept free ranging in a fenced field about 200 m². Thirty-nine died over a few days, with necropsy showing typical signs of RHD: epistaxis, profuse bloody effusion in the thoracic and abdominal cavities, tracheal congestion, lung haemorrhages, severe degeneration and discoloration of the liver, and splenomegaly.

Negative staining electron microscopy (IEM) was performed from liver homogenates as previously described (Lavazza et al., 1996). Several virions, morphologically resembling lagovirus, were observed. The presence of RHDV was then confirmed by RT-PCR. Briefly,

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total RNA was extracted from the livers of three necropsied rabbits by using the RNeasy Mini Kit (Qiagen, Milan, Italy). RT-PCR was performed by using the SuperScript III One-Step RT-PCR System (Life Technologies, Milan, Italy). The sequence of primers (RHDVgf, 5'-AATGTTATGGAGGGCARAGCC-3'; RHDVgr, 5'-AGRCCAGGGTC RCCRGTGGRTG-3') was based on a conserved region of the VP60 gene, encoding for the capsid protein of RHDV. The 563 bp amplicons gathered from the three samples were cloned and sequenced. Each had the same nucleotide sequence, and a representative was submitted in GenBank with accession no. KC741408. The BLAST analysis of sequences (excluding primers) revealed they were 97% (99% at amino acid level) identical to those of the corresponding fragment of the VP60 gene of the RHDV2, isolate Ud11 (accession no. JQ929052), which was identified in Italy on mid 2011 (Le Gall-Reculé et al., 2013).

About 30 m from the rabbit enclosure, 30 Italian hares (*Lepus corsicanus*) were reared in a fenced unit. Rabbits and hares were physically separated, so no direct contacts occurred between them, but indirect contacts could occur, as the same operators managed both groups.

The hare group consisted of 20 adults and 10 young animals (less than 6 months old), kept in outdoor cages 6 m², and each cage hosted two hares. They were reared within the Sicilian Region and University of Palermo Program for Italian hare, for the study of *L. corsicanus*. The species is considered globally vulnerable and is endangered in Italy (IUCN, 2008). The reared hares were identified as *L. corsicanus* according to the morphological and phenotypic criteria of Riga et al. (2001) and Ruge et al. (2009). During the RHD outbreak occurring in the adjacent rabbitry, a hare died, without visible signs other than epistaxis. At necropsy there was heavy pulmonary and tracheal oedema with profuse haemorrhages and a slightly enlarged, congested liver (Fig. 1A). The tracheal lumen was also haemorrhagic with profuse foamy exudate (Fig. 1B). There were no signs of enteric or neurological disorders. Routine bacteriological and parasitological tests were negative, as was the EBHSV-specific RT-PCR (Ros Bascuñana et al., 1997). The RT-PCR with the RHDVgf/RHDVgr primer pair returned the 563 bp amplicon. The RT-PCR product was cloned and sequenced and was found to be 100%

identical to those retrieved from rabbits, thus demonstrating that rabbits and the Italian hare were infected by the same RHDV2 strain.

To definitively confirm the RHDV2 infection of the rabbits and the Italian hare, the antigenic profile of the virus was assessed by testing the livers and spleen homogenates of the three necropsied rabbits and the hare with a sandwich ELISA employing a large panel of anti-RHDV MABs produced against RHDV, RHDVa and RHDV2, as previously described (Le Gall-Reculé et al., 2013; Puggioni et al., 2013). The reactivity pattern of the virus from rabbits and hare showed the typical profile for RHDV2, being unreactive with the MABs specific for the classical RHDV (strain Bs89) and RHDVa (strain Pv97), but reactive with the two new MABs specific for RHDV2 (data not shown).

In order to extend the analysis of the RHDV2 strain infecting the Italian hare, the entire VP60 gene was amplified as previously described (Le Gall-Reculé et al., 2013) and sequenced (GenBank accession no. KC741409). It showed 97.2% of nucleotide identity and 98.8% of amino acidic identity with the same gene of the RHDV2 strain Ud11. To perform phylogenetic analysis, the nucleotide sequence from the identified virus (named Sr12) was aligned by ClustalW with the VP60 gene sequences available in GenBank. The sequence panel included the G1-G6 RHDV genogroups, non-pathogenic rabbit calicivirus (RCV) and RHDV2 strains, as listed in Table S1. The Neighbor-Joining phylogenetic tree was generated by using the Kimura 2-parameter evolutionary model, implemented in MEGA 6 (Tamura et al., 2013). Bootstrap resampling was performed on 1,000 replicates.

The phylogenetic tree (Fig. 2) evidenced that the Sr12 RHDV2 clustered together with the previously identified RHDV2 strains, which formed a distinct clade, separated by the RHDV and RCV groups. Interestingly, Sr12 was distinct, though closely related, to the other strains from Northern Italy, Sardinia and France. All together, these data demonstrated that the RHD outbreak was caused by the emerging lagovirus RHDV2.

Interestingly, other than rabbits, the same viral strain infected an Italian hare and induced a RHD-like disease, further supporting evidence of the capability of RHDV2 to infect *Lagomorpha* hosts other than *O. cuniculus*. While the host restriction of the

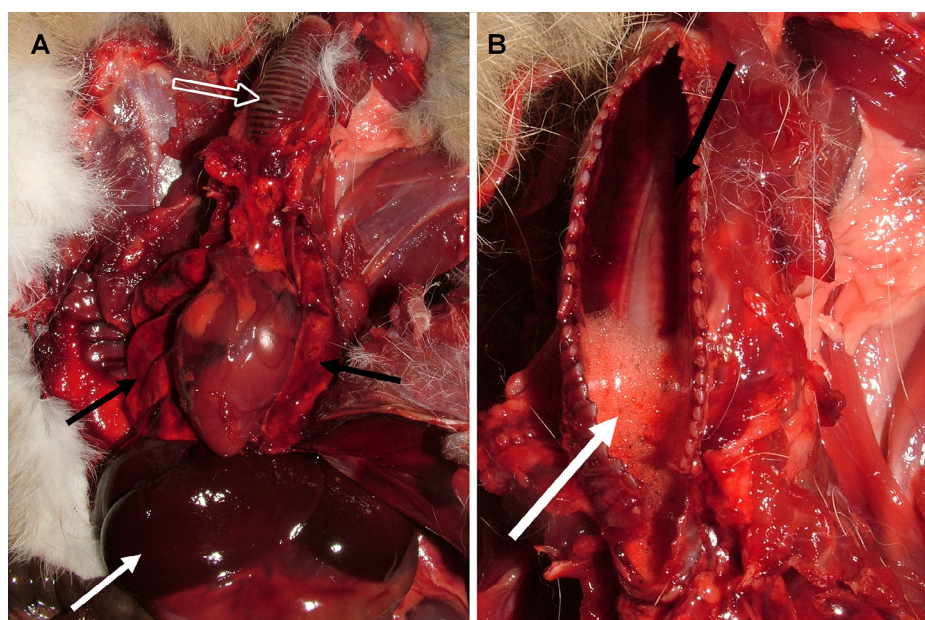


Fig. 1. Gross lesions of the Italian hare. A) Diffuse hyperaemia on the tracheal wall (empty arrow) and on the lungs (black arrows). Liver is congested (white arrow). B) Trachea. Extensive mucosal hyperaemia (black arrow) and foamy exudate (white arrow) in the lumen.

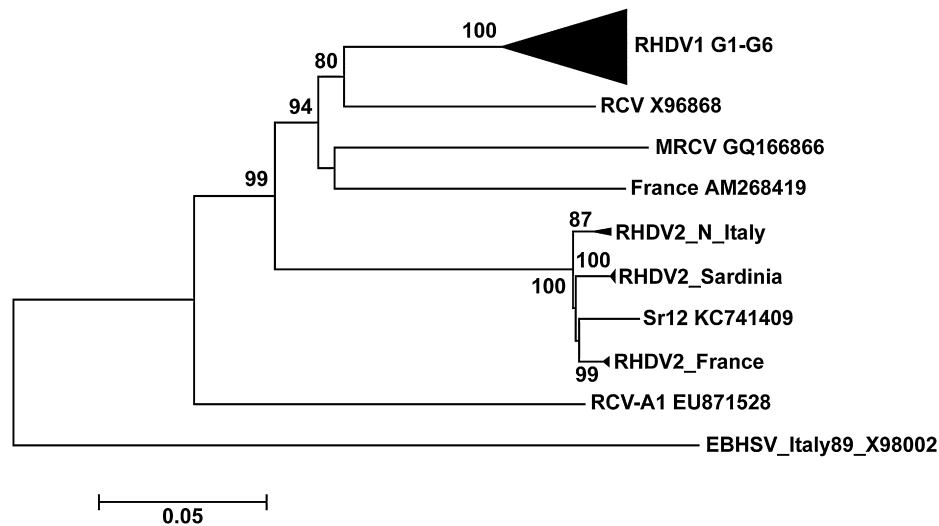


Fig. 2. Phylogeny of the RHDV strains, identified by their GenBank accession number and their strain code. Bootstrap values above 70% are indicated at the nodes.

“classical” RHDV and the EBHSV remains well established (Lavazza et al., 1996) and unquestionable to date, recent data are progressively revealing that some lagoviruses, and RHDV2 in particular, may have a wider host range (Puggioni et al., 2013).

To verify the extent of circulation of virus among the hares, three months after the outbreak serological tests were performed on 18 out of them, including that living in the same cage of the one infected. ELISA competition tests were performed as described in Capucci et al. (1991). Briefly, sera were diluted from 1/10 on ELISA plates previously adsorbed with hare or rabbits immune sera, specific for EBHSV, RHDV and RHDV2, respectively. The proper specific antigen was added, together with the sera, to each plate at a pre-established dilution. Finally, horseradish peroxidase labeled monoclonal antibodies (MAbs) specific for each of the three virus were added to the plates in order to semi-quantify the binding of virus to the wells. The threshold value negative/positive was equal to the 75% value of the negative controls at the 1/10 dilution. Results showed that all sera were negative for antibodies against the three viruses tested.

In this outbreak all the other hares survived, including the hare present in the same cage of the dead one, and the serologically tested animals were negative for both RHDV2 and EBHSV. The reasons of such a low prevalence and mortality in hares, compared to the high mortality among rabbits, could only be speculated. Considering long-term persistence of antibodies induced by lagovirus infection in hares and rabbits (Cooke et al., 2000), the complete seroconversion from positive to negative seems unlikely in all hares within a short time.

Therefore, the most possible explanation is that most hares could have been contaminated by RHDV2 but without becoming infected. This would imply a reduced susceptibility of Italian hares to RHDV2, at least when they are healthy, normoreactive and fully immunocompetent.

A second possibility is that RHDV2 had had a limited circulation among hares, due to the separations and the physical barriers existing among themselves and with respect to rabbits. However this hypothesis seems less probable because even the animal living together with the dead hare resulted seronegative and completely asymptomatic.

Those results infer that the virulence of RHDV2 is reduced in *L. corsicanus* when compared with the far higher mortality observed in *O. cuniculus*. Thus, the level of susceptibility to RHDV2 of *L. corsicanus* should be intermediate between that of Sardinian cape

hare (*L. capensis mediterraneus*), in which RHDV2 infection was similar in terms of diffusion and severity to that observed in wild rabbits, and that of European brown hare (*Lepus europeus*). In fact, on the basis of epidemiological data, it has been suggested that the European brown hare is either not susceptible, or less susceptible to RHDV2 (Puggioni et al., 2013). This behavior toward RHDV2 infection may have a genetic origin, since, as shown by Pierpaoli et al. (1999) who investigated the genetic variations among hare populations of the Italian peninsula and Sicily, the Italian hare is genetically distinct and deeply divergent from the other Eurasian and African hares. In addition, since the Italian hare is most genetically related to the mountain hare (*Lepus timidus*), which is present in reduced population on the Alps, it would be interesting to implement surveillance and/or experimental studies to verify its potential natural susceptibility to RHDV2.

In conclusion, the field evolution and circulation of RHDV2 should be strictly monitored to promptly assess if it might be capable of infecting other lagomorphs. In fact, the potential to infect more widely distributed species, such as the *Lepus europaeus*, might lead to a wider and more rapid spread of RHDV2 and, at the same time, seriously threaten vulnerable or endangered *Leporidae* species. Thus, further studies should be aimed to ascertain if the peculiar capsid structure of RHDV2 may have facilitated the attachment and/or binding, the invasion and the subsequent cellular stages of viral cycle, thus paving the way to the virus for infecting new hosts.

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Conflict of interest

None to declare.

Appendix: Supplementary Material

Supplementary data to this article can be found online at [doi:10.1016/j.rvsc.2014.10.008](https://doi.org/10.1016/j.rvsc.2014.10.008).

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