STUDY OF GENETIC EVOLUTION OF RHDV2 IN ITALY FROM 2011 TO 2019

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ABSTRACT

Rabbit Haemorrhagic Disease Virus (RHDV) is a very virulent virus of the genus *Lagovirus* causing a severe and fatal hepatitis in the European rabbit (*Oryctolagus cuniculus*), with 100% morbidity and 80-95% mortality. Its firstly emerged in 1984 in China and then it rapidly diffused worldwide in those countries where the European rabbit is present. On 2010 a new RHDV-related virus, called RHDV2, showing a specific antigenic profile different from RHDV, emerged in Europe. It again rapidly spread worldwide becoming prevalent in the field and causing extended epidemics in wild and domestic rabbits and also in some hare species. Indeed, since the first identification, RHDV2 virulence increased and it frequently underwent to recombination events.
To understand the virus evolution in Italy, we sequenced the capsid gene plus an 800bp upstream of the start codon, of 87 RHDV2 strains identified from 2011 to 2019. The phylogenetic analysis showed that the different Italian isolates fall in the same cluster of other RHDV2 strains identified in Europe. In particular, they appear to be divided into subgroups more related to the identification year than to geographical origin, with the exception of three strains identified respectively in 2013-14 in Cuneo and Perugia province and in Sardinia in 2016, located in the subgroup of the viruses firstly identified in France and Italy in 2010-2011. In addition, we detected 10 recombinant strains that show the break point located in a region close to the VP60 initiation codon and include the RHDV2 structural proteins with RHDV-G1 non-structural proteins. Considering that the RHDV genotype G1 circulated in the Iberian Peninsula until the appearance of RHDV2 and it is now completely disappeared, while in Italy the G6 and G3 RHDV genogroups are still circulating, we could presume that such recombinant strains more likely originated in Portugal/Spain and they were then "introduced" in Italy, an hypothesis supported by the phylogeography analysis.

Key words: *lagovirus*, recombination, phylogeography, evolution.

INTRODUCTION

Rabbit Haemorrhagic Disease Virus (RHDV) is a very virulent virus of the genus *Lagovirus* causing a severe and fatal hepatitis in rabbits (*Oryctolagus cuniculus*), with an incubation of 36-48 hours, 100% morbidity and 80-95% mortality (Abrantes et al., 2012). After its emergence in China in the 80s it rapidly diffused to those countries where European rabbit was present. Then, a new RHDV-related virus, called RHDV2, emerged in Europe on 2010 causing large epidemics and severe losses to rabbit domestic and wild populations, and again it rapidly spread worldwide (Le Gall-Reculé et al., 2011 and 2013). Thanks to its specific antigenic profile, allowing to largely escape the heard immunity previously generated by RHDV, RHDV2 became prevalent in the field causing extended epidemics in wild and domestic rabbits, and affecting also some hare species (Velarde et al., 2016). Indeed, since the first identification, RHDV2 virulence increased (Capucci et al.; 2017) and it frequently underwent to recombination events (Lopes et al.; 2015), but it is not clear if such frequency of recombination is a peculiarity of RHDV2 or rather more generally of *lagoviruses*, similar to other ssRNA viruses. However, its occurrence, likely linked to the complex RHD epidemiology, involving at the same both domestic and farmed rabbits and large populations of wild animals, could have been underestimated in the past.
RHDV2 could not be considered a simple evolution (a direct variant) of RHDV but rather a new serotype, originated from an unknown source. Unlike RHDV, the available data suggest that RHDV2 emerged and evolved in Europe. Regardless the origin of RHDV2 (for recombination among

lagoviruses? for a species jump?), is well known that an emerging virus evolves searching for its "best fitness". This process could take several years, actually never stops, and it could be initially characterized by continuous variation in the phenotype of the virus (i.e. pathogenicity, virulence, antigenic profile). In addition, such process for RHDV2 could be split into single-sub processes within different countries, since the "original" RHDV2 isolates have then evolved in different epidemiological contexts, mainly characterized by the presence of both different *lagoviruses* and hosts.

Objectives of this study are: a) to evaluate the phylogenesis of RHDV2 strains detected in Italy, also in comparison with those detected in other European countries; b) to check the presence of recombinants strains, including the RHDV2 Iberian–like recombinants or even new "Italian recombinants".

MATERIALS AND METHODS

Samples

We have examined 87 strains, selected from more 300 RHDV2 strains, identified in Italy between 2011-2019 from wild and domestic rabbits, stored as liver homogenates, at 10% v/w in PBS with 50% of glycerol and conserved at – 24° C. The selection was made on geographical (area of origin) and temporal (year of identification) criteria.

RNA extraction, RT-PCR amplification and sequence analysis

The virus RNA was extracted from the liver homogenates and the vp60 gene amplified by RT-PCR as previously described (Le Gall et al., 2012). To preliminary detect putative recombinants strains, a specific RT-PCR assay has been developed to amplify only a portion of the region encoding the non structural proteins (NSP) of RHDV2. The primers were designed by alignment all the full genome sequences of *lagoviruses* available in GenBank.

To sequence the entire genome five overlapping amplicons were obtained, gel purified and sequenced. The 3'-terminal sequence were determined using an Oligo (dT)-Adapter primer flanked by an adapter sequence for the cDNA synthesis and the primer adapter was used in RT-PCR and sequencing reaction. The 5'-terminal sequence was determined using a 5' Race commercial kits. Conting assembling and genome sequence analysis was carried out by Seqman NGen DNASTAR version 11.2.1 (DNASTAR, Madison, WI, USA) and recombination analysis verified by Symplot and RdP4 software comparing the RHDV2 sequences with *lagovirus* sequences present in GenBank. The computer programmes Megaligne (DNASTAR Lasergene 10 Core Suite) and MEGA 6.0 will be used for sequence alignments and phylogenetic trees.

Phylogeography

To explore the origin of outbreak and the geographical distribution of the RHDV2 virus, we used a probabilistic model of evolution based on Bayesian Markov Chain Monte Carlo (MCMC) method implemented in BEAST package (v 1.10) (Nascimento et al., 2017), phylogenetic and phylogeographic software that allow various evolutionary models to be tested with different model and substitution rate parameters, using a set of genetic sequences obtained over time in a specific geographic area. The *vp60* gene sequences were analyzed using HKY nucleotide model substitution SRD06 parametrization , with gamma heterogeneity site model; for the BEAST analysis uncorrelated relax normal clock (UCLD) was used with lognormal distribution, and assume as prior constant population coalescent size over the time

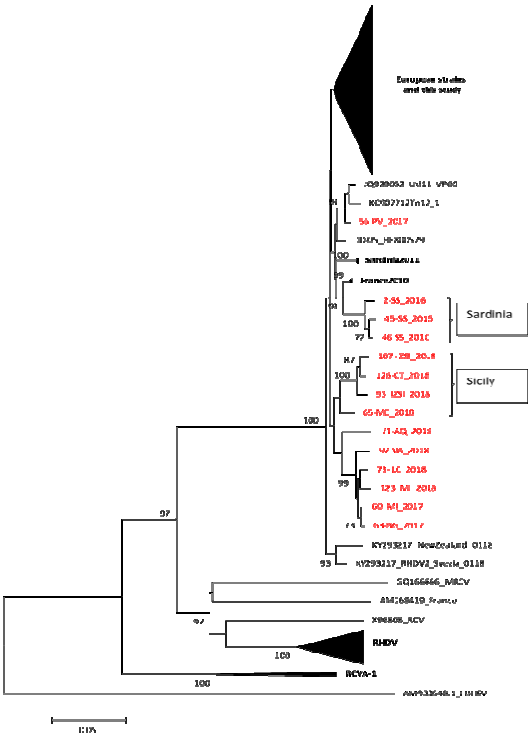
The geographic location was assigned to each sample as a discrete trait, and separate data partition was created for this trait. The symmetric discrete trait substitution model was assigned to parameter complexity, and ancestral state reconstruction was applied to the trait partition (Li et al., 2011). Each BEAST analysis was run for million generation until convergence was achieved. The BEAST output was analyzed in TRACER software, and the maximum clade credibility (MCC) tree was created using TreeAnnotator program, for the posterior set of trees (Lemey, et al., 2009). The phylogeographic diffusion was analyzed using discrete trait in Spread software, and the output was viewed in Google earth, a spatial projection of the genetic lineages based on their phylogeographic relationships.

RESULTS AND DISCUSSION

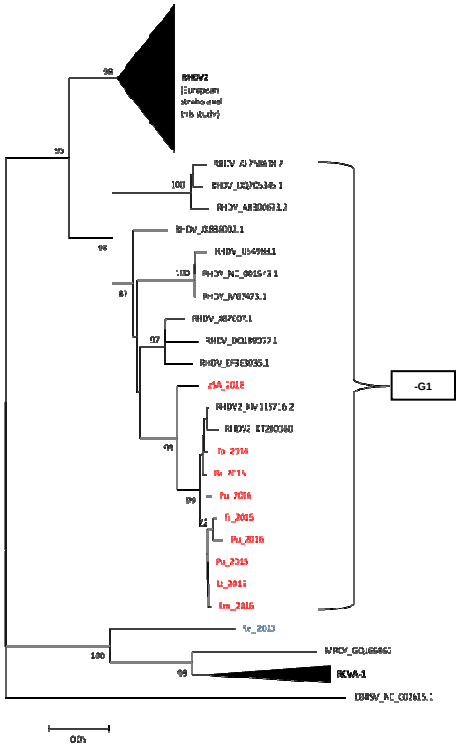
A fragment of 2.5 Kb was amplified and sequenced for 87 RHDV2 strains identified in Italy from 2011 to 2019. A phylogenetic tree inferred for the capsid sequences, including publicly available RHDV sequences of G1–G6, as well as non-pathogenic *lagoviruses*, showed that the majority of the strains fall in the same cluster of RHDV2 identified in Europe. Apparently the Italian strains don't belong to a specific subgroup related to the year of identification or geographical origin, with the exception of some strains from North of Italy, Sardinia and Sicily that are located in the subgroup of the viruses firstly identified in France and Italy in 2010-2011 (Figure 1 Panel A).

Following the observation of a recombination breakpoint in the 5' region of the capsid gene in the recently characterized RHDV2 recombinant strains (Lopes et al., 2015b), we have also reconstructed the phylogeny based on the fragment sequenced upstream of the capsid gene VP60. Phylogenetic analysis (Figure 1 Panel B), showed nine recombinant strains that have the break point located in a region close to the vp60 initiation codon and include the RHDV2 structural proteins with RHDV-G1 non-structural proteins. Considering that the RHDV genotype G1 circulated in the Iberian Peninsula until the appearance of RHDV2, and now it is completely disappeared, while in Italy the G6 and G3 RHDV genogroups are still circulating, we could presume that such recombinant strains more likely originated in Portugal/Spain and then they have been "introduced" in Italy, as confirmed by phylogeography study.

A)



B)



C)

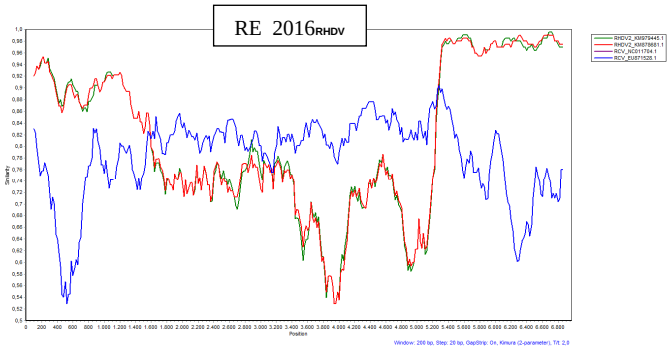


Figure 1: A neighbor-joining phylogenetic tree was generated by using the Kimura 2 parameters evolutionary model implemented in MEGA 6 using vp60 gene (panel A) or the non-structural portion of the genome (panel B). It is also shown the symplot analysis performed on the full genome of sample RE_2016 (panel C). Bootstrap analysis was performed using 1,000 replicates.

Interestingly, we also found a recombinant strain (Re_2016) that presents the 5' end of the genome from an RHDV2 strain, the non structural portion of the genome from RCV-E2 and the structural portion of the genome from RHDV2 (Figure 1 Panel C). In this case, beacuse RCV-E2, a non pathogenic rabbit calicivirus, it is still circulating in Italy, it is possible to hypothesize that this recombinant strain generated in Italy.

For the phylogeography analysis based on the vp60 sequences, it appears that the first RHDV2 strain arrived in Italy from France on 2011, initailly to the north-eastern Udine province, than it moved to Sardinia Island, and from there it finally spread to the whole country. During the following years there was also a further introduction of strains from the Iberian Peninsula (e.g. NA_2015) and interestingly some strains (e.g. SS_2016) were again introduced from France to Italy (Figure 2).

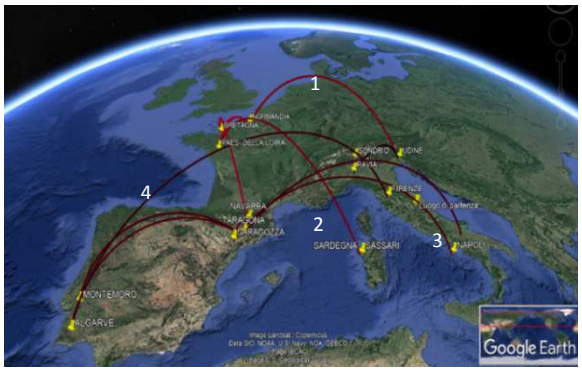


Figure 2: Phylogeographic dispersion of RHDV2 in Europe. The cartographic plane referring to the regions of study. The dispersion lines are indicated according to a time-related color gradient, where red refers to the minimum time and dark red to the most recent time. The numbers indicate the time sequence with which RHDV2 probably arrived in Italy (1: 2011, 2: 2012, 3:2015, 4: 2016).

CONCLUSIONS

Based on the analysis conducted in this study, RHDV2 seems to be initially arrived from France (first place of virus identification), and then, more recently, introduced from the Iberian Peninsula. Once arrived in Italy, the virus spread throughout the peninsula without apparently giving rise to space-time clusters.

We have identified some recombinant viral strains, and the fact that the recombinant viruses found have spread and are therefore vital depends on the high structural homology of the parental strains (RHDV, RHDV2, RCV) that are the subject of the recombination process. The recombination event in RHDV2 and in general in *lagoviruses*, could have a much higher frequency than that highlighted until now, due to the high homology among the circulating strains of RHDV/RHDV2 and therefore the impossibility to distinguish parental strains from recombinants.

ACKNOWLEDGEMENTS

This research was supported by Italian Ministry of Health, project PRC2017/002 to IZSLER

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