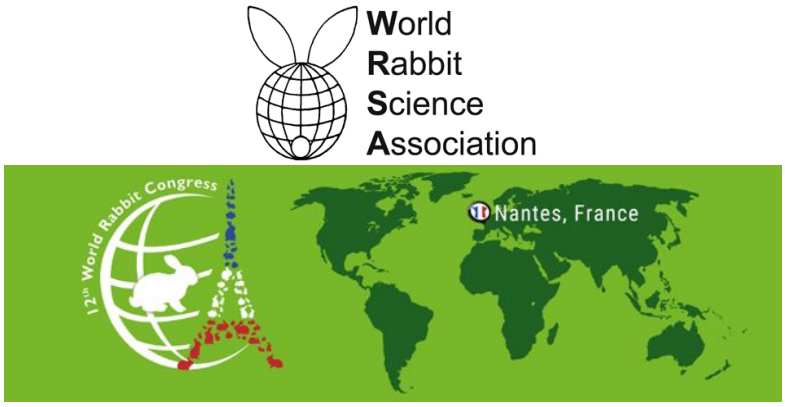


PROCEEDINGS OF THE 12th WORLD RABBIT CONGRESS

Nantes (France) - November 3-5, 2021
ISSN 2308-1910

Session
PATHOLOGY & HYGIENE

Cavadini P., Campisi G., Vismara A., Lavazza A., Capucci L.

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

Full text of the communication
+
Slides of the oral presentation

How to cite this paper
Cavadini P., Campisi G., Vismara A., Lavazza A., Capucci L., 2021. Study of genetic evolution of RHDV2 in Italy from 2011 to 2019. Proceedings 12th World Rabbit Congress - November 3-5 2021 - Nantes, France, Communication P-11, 4 pp. + presentation

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

Cavadini P. , Campisi G. , Vismara A. , Lavazza A. [*] , Capucci L.

Dept. of Virology, IZSLER, Via Bianchi 9, 25124, Brescia, Italy
[*]Corresponding author: antonio.lavazza@izsler.it

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

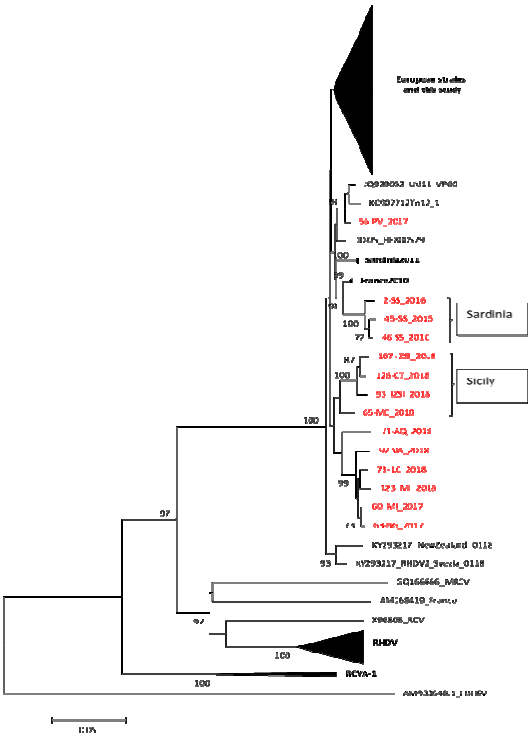
The geographic location was assigned to each sample as a discrete trait, and separate data partition was created for this trait. The symmetric discrete trait substitution model was assigned to parameter complexity, and ancestral state reconstruction was applied to the trait partition (Li et al., 2011). 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

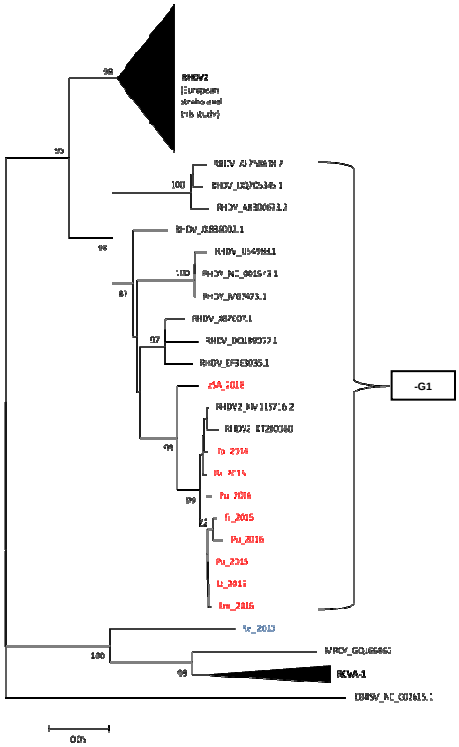
A fragment of 2.5 Kb was amplified and sequenced for 87 RHDV2 strains identified in Italy from 2011 to 2019. A phylogenetic tree inferred for the capsid sequences, including publicly available RHDV sequences of G1–G6, as well as non-pathogenic *lagoviruses*, showed that the majority of the strains fall 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)

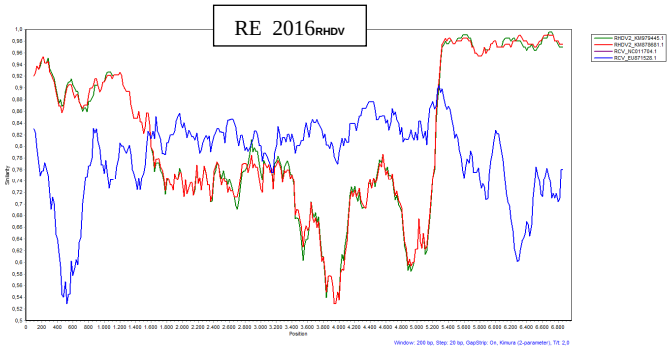


Figure 1: A neighbor-joining phylogenetic tree was generated by using the Kimura 2 parameters evolutionary model implemented in MEGA 6 using vp60 gene (panel A) or the non-structural portion of the genome (panel B). It is also shown the symplot analysis performed on the full genome of sample RE_2016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

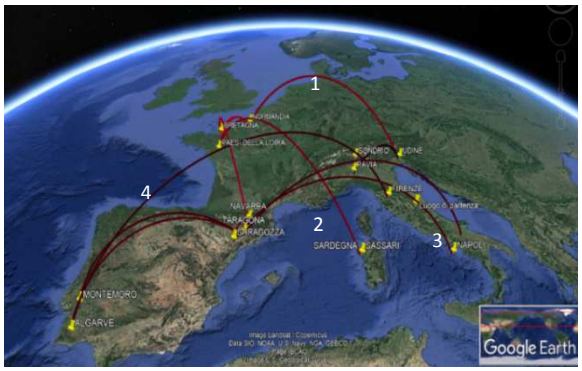


Figure 2: Phylogeographic dispersion of RHDV2 in Europe. The cartographic plane referring to the regions of study. The dispersion lines are indicated according to a time-related color gradient, where red refers to the minimum time and dark red to the most recent time. The numbers indicate the time sequence 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

REFERENCES

Abrantes, J., Van Der Loo, W., Le Pendu, J. & Esteves, P.J. (2012) Rabbit haemorrhagic disease (RHD) and rabbit haemorrhagic disease virus (RHDV): a review. Veterinary Research 43, 12-31

Capucci, L., Cavadini, P., Schiavitto, M., Lombardi, G., Lavazza A. (2017) Increased pathogenicity in rabbit haemorrhagic disease virus type 2 (RHDV2). Vet Rec. 29, 180 (17):426

Le Gall-Reculé, G., Lavazza, A., Marchandeau, S., Bertagnoli, S., Zwingelstein, F., Cavadini, P., Martinelli, N., Lombardi, G., Guérin, J.L., Lemaitre, E., Decors, A., Boucher, S., Le Normand, B. & Capucci, L. (2013) Emergence of a new *lagovirus* related to Rabbit Haemorrhagic Disease Virus. Veterinary Research. 44, 81-94.

Lemey, P., Rambaut, A., Drummond, A. J., & Suchard, M. A. (2009) Bayesian phylogeography finds its roots. PLoS computational biology, 5(9), e1000520.

Lopes, Am., Dalton, K.P., Magalhães, M.J., Parra, F., Esteves, P.J., Holmes, E.C., Abrantes, J. (2015) Full genomic analysis of new variant rabbit hemorrhagic disease virus revealed multiple recombination events. J Gen Virol. 96, 1309-1319.

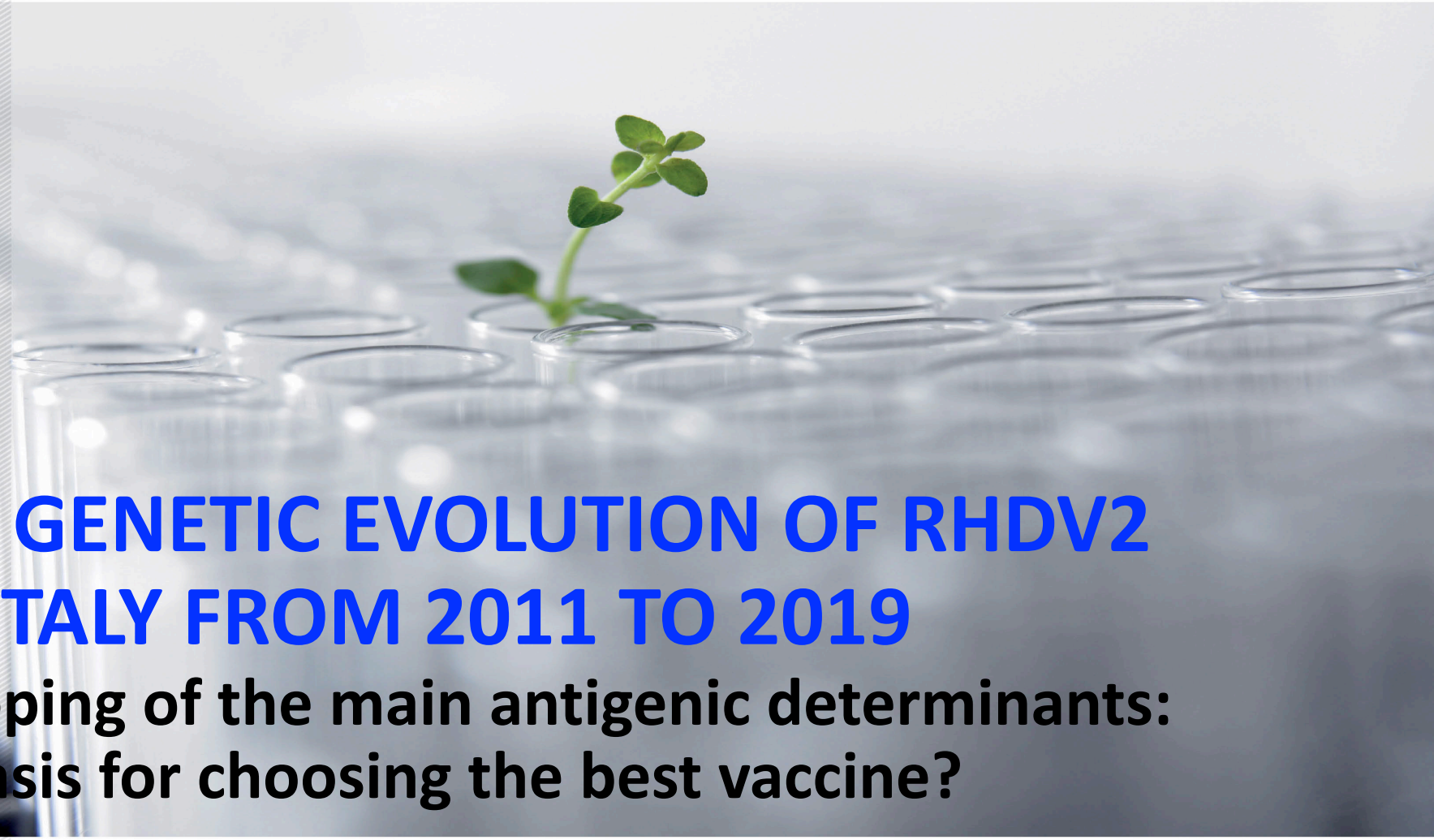
Nascimento F.F., Dos Reis M. And Yang Z. (2017) A biologist's guide to Bayesian phylogenetic analysis. Nat Ecol Evol. October; 1(10): 1446–1454.

Velarde, R., Cavadini, P., Neimanis, A., Cabezón, O., Chiari, M., Gaffuri, A., Lavín, S., Grilli, G., Gavier-Widén, D., Lavazza, A., Capucci, L. (2016) Spillover Events of Infection of Brown Hares (Lepus europaeus) with Rabbit Haemorrhagic Disease Type 2 Virus (RHDV2) Caused Sporadic Cases of an European Brown Hare Syndrome-Like Disease in Italy and Spain. Transbound Emerg Dis. doi: 10.1111/tbed.12562

Li, W.L.S.., Drummond, A.J.. (2011) Model Averanging and Bayes Factor Calculation of relaxed Molecular Clocks in Bayesian Phylogenetics; mol. Bio. Evol. 29(2):751-761.2012

WRSA - 12th World Rabbit Congress -
November 3-5, 2021 - Nantes, France

ISTITUTO ZOOPROFILATTICO SPERIMENTALE
DELLA LOMBARDIA E DELL'EMILIA ROMAGNA
"BRUNO UBERTINI"
ENTE SANITARIO DI DIRITTO PUBBLICO
OIE Reference Laboratories for Rabbit
Haemorrhagic Disease and for Myxomatosis



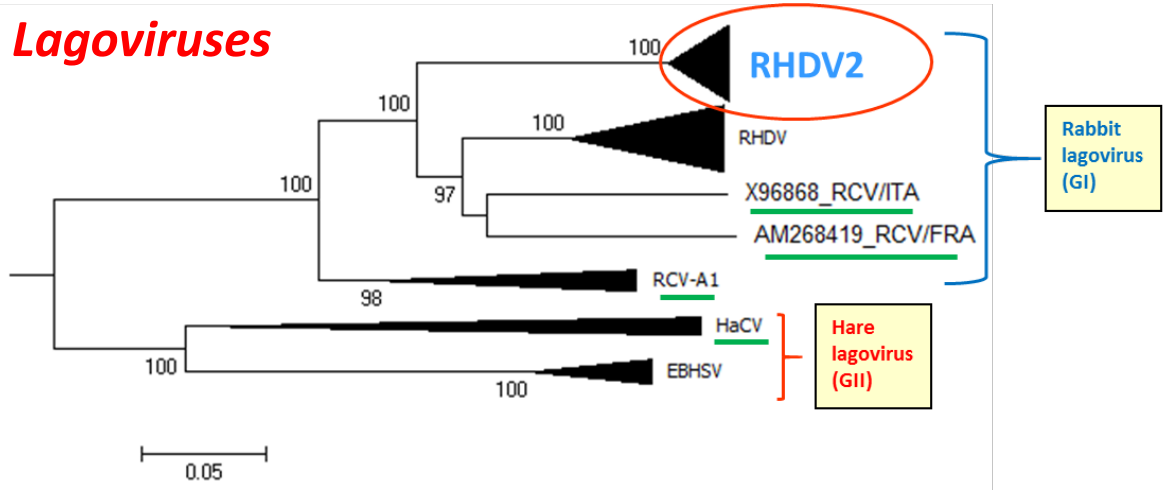
STUDY OF GENETIC EVOLUTION OF RHDV2 IN ITALY FROM 2011 TO 2019

Detailed mapping of the main antigenic determinants: the basis for choosing the best vaccine?

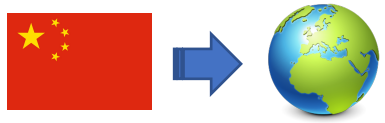
Cavadini P., Campisi G., Vismara A., Lavazza A. and Capucci L.

Rabbit Haemorragic Disease

- Severe necrotic hepatitis
- High mortality rate (80-90%)
- Caused by a RNA SS+ virus **RHDV**
- Genus *Lagovirus*, Family Caliciviridae



~ 1980-85



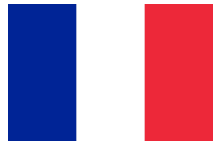
Rabbit Haemorrhagic Disease RHDV – GI.1

Wild and farmed rabbits
Oryctolagus cuniculus

European Brown Hare Syndrome EBHSV – GII.1

Brown hare
(Lepus europaeus P.)

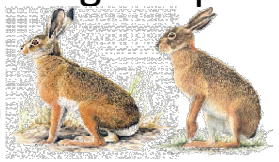
2010 – France

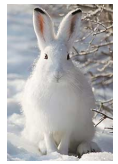


RHDV2 – GI.2

a ”new” **serotype** with distinct genetic and antigenic profile to RHDV





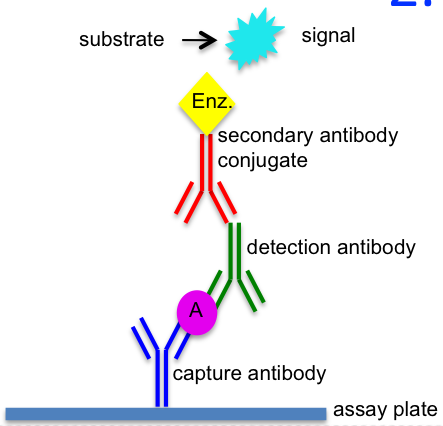



Antelope rabbit (*Lepus alleni),*
Desert cottontail (*Sylvilagus audubonii*)
Mountain cottontail (*Sylvilagus nuttallii*)
Eastern cottontail (*Sylvilagus floridanus*)

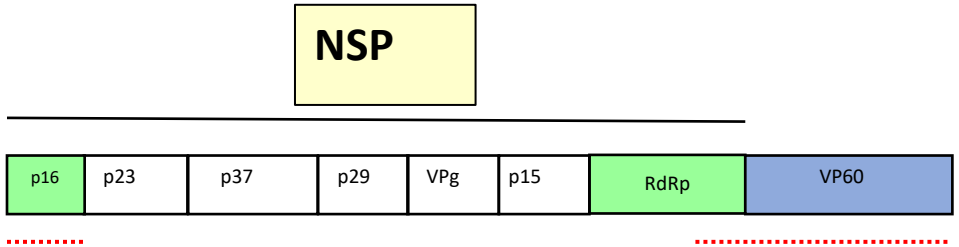
Aims and design of the study

- Study the phylogenesis of RHDV2 strains identified in Italy since first identification (summer 2011) to today
- Identify the different RHDV2 recombinants circulating in our country
- Locate the major antigenic determinants on the surface of the virus

1. Phylogenetic analysis of **165** RHDV2 strains selected from over **300** strains from different areas of ITALY, identified during the period 2011-2019



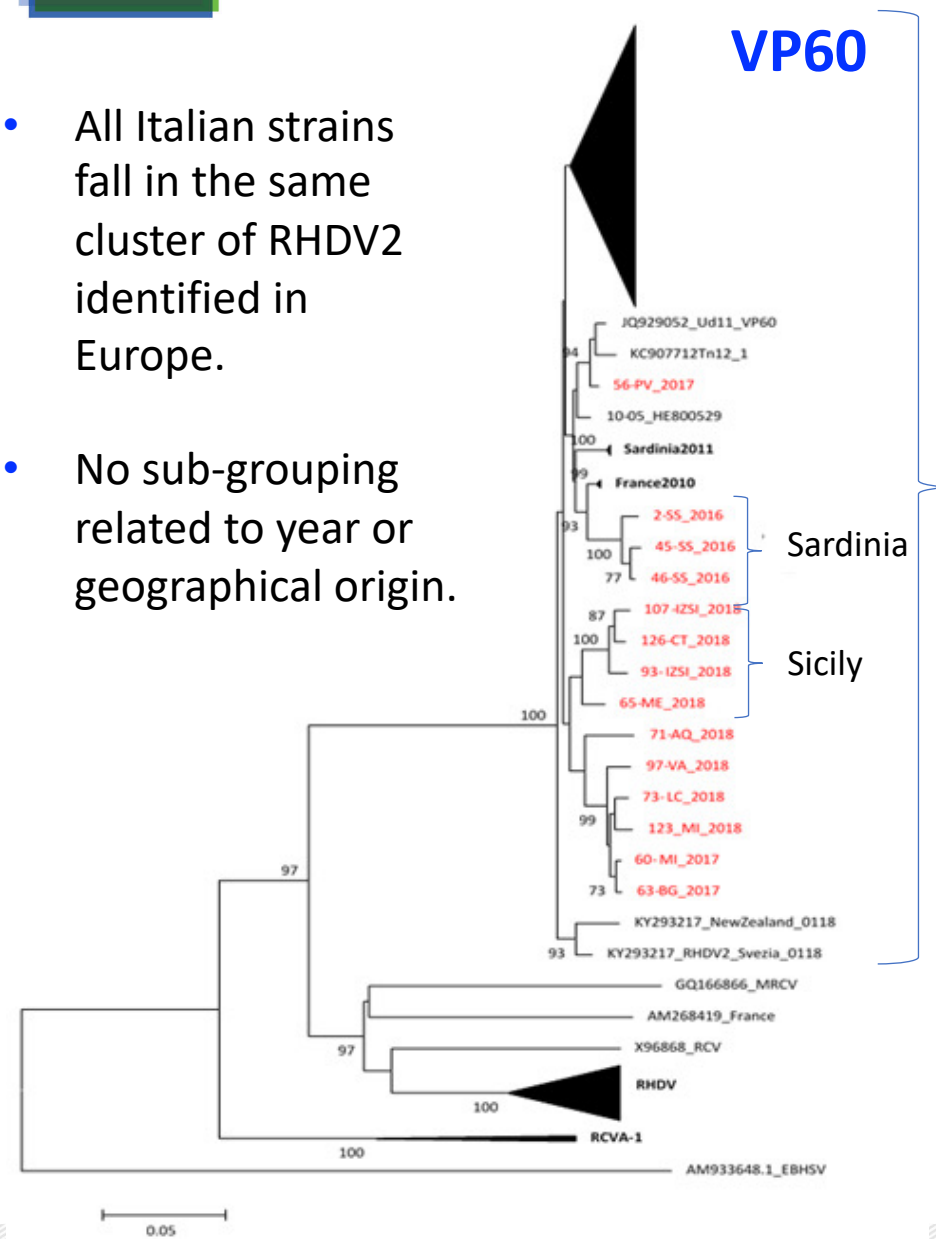
2. Sequence of both the capsid gene (VP60) and a portion of non structural proteins (p16 and RdRp)

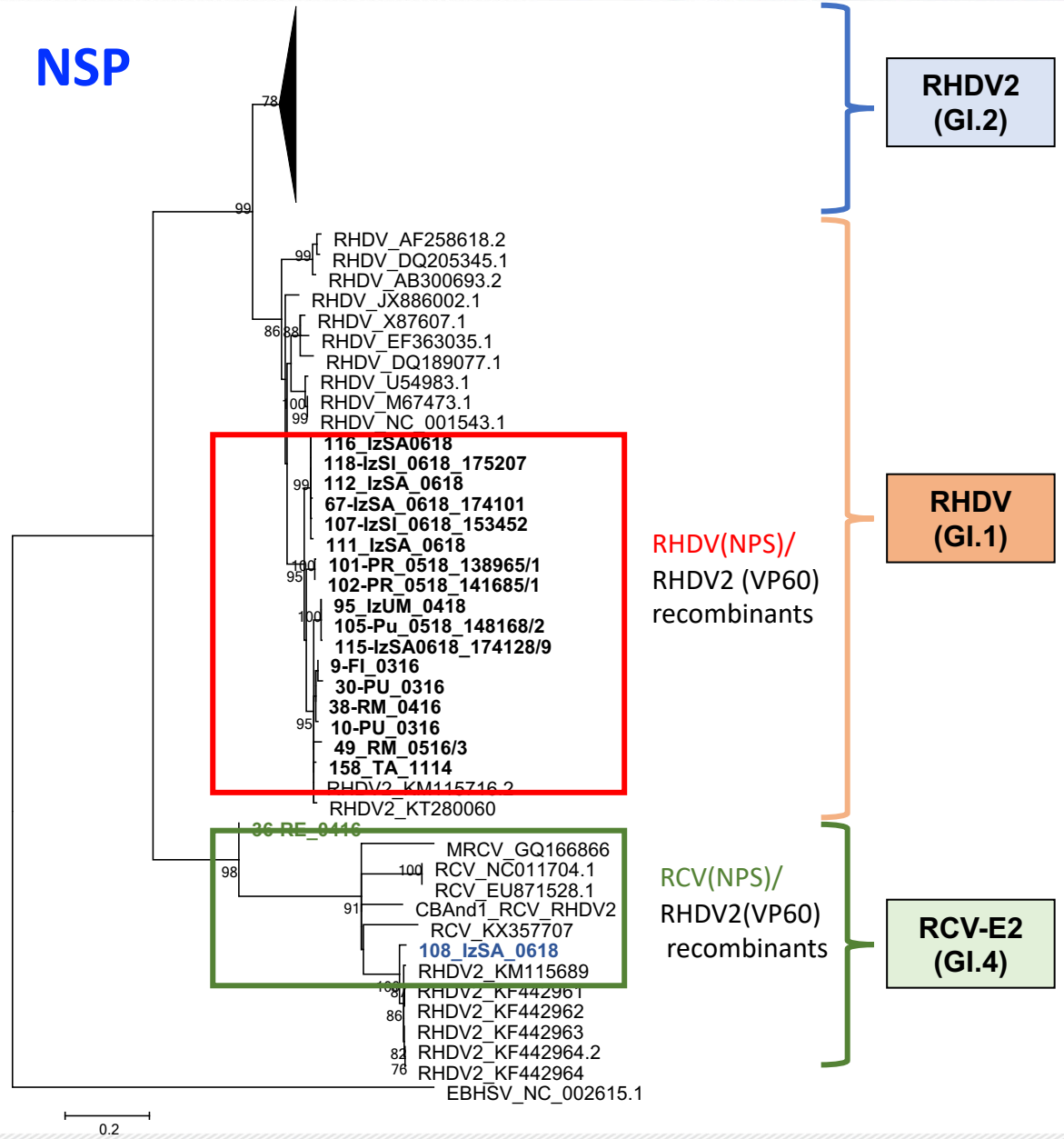


3. ELISA assay with 16 mAbs against RHDV2

Results: phylogenetic analysis

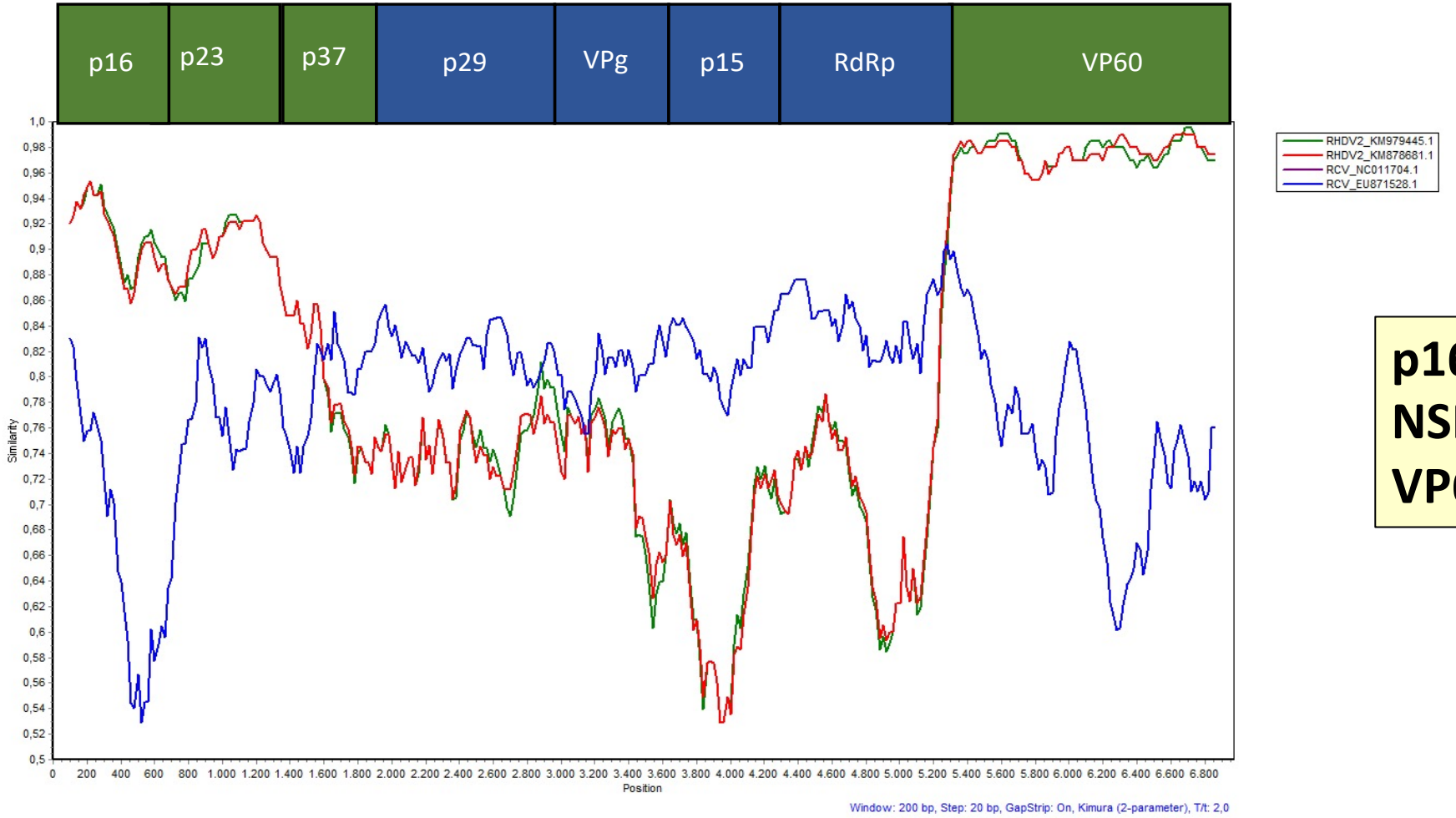
- All Italian strains fall in the same cluster of RHDV2 identified in Europe.
- No sub-grouping related to year or geographical origin.





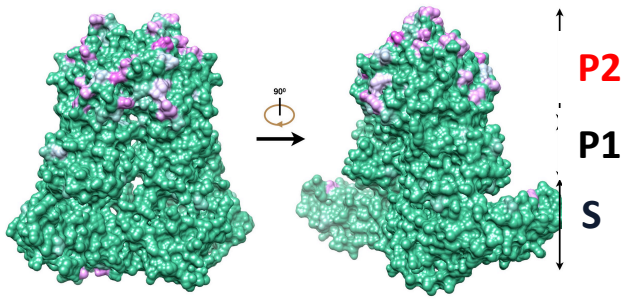
Results: RCV-E2 (GI.4)/RHDV2 «double» recombinant

Strain: 36-RE_0416



p16+p37: 90% **R2**
NSP:90% **RCV-E2**
VP60: 98% **R2**

Results: antigenic determinants



- Panel of **16 mAbs** against RHDV2 (2010 and 2014 strains)
- Analysis of more than **300 strains**
- Strains grouped based on % of reactivity

Epitope	ELISA Reactivity
Fully conserved	100%-70%
Partially conserved	70%-15%
Lost	<15%

Epitope mapping throughout the comparison of the VP60 aa sequence of selected strains

Results: MAbs reactivity

Strains used for Mabs production

First highly pathogenic strain

Early strains

Vaccine strains

MAb	4D2	4H12	1C6	1F8	3F6	4B4	2B11	4D6	4C5	3C10	4F7	1C10	3G9	4H7	2G5
FRA1032	-1	-2	74	92	102	103	97	95	34	83	75	99	95	71	95
TV14	95	76	1	2	2	-1	9	42	3	20	93	103	98	106	79
FE10.13	-2	-1	95	1	102	2	94	97	6	93	100	102	101	99	107
BG12.15	-1	4	95	2	-1	0	101	95	2	95	100	100	99	95	104
MI10.17	82	0	3	1	0	0	8	22	4	8	102	99	99	99	82
MI05.14	-2	95	105	1	-1	-1	121	98	8	100	100	104	99	100	102
ME05.18	1	109	37	0	1	1	10	4	4	5	3	90	108	4	24
MI04.16	100	101	3	2	-1	-1	12	13	-1	0	104	113	82	97	4
VAX 1	0	130	0	109	2	-2	99	96	3	100	100	109	111	93	96
VAX 2	0	-1	101	92	103	101	96	98	101	84	90	102	96	79	102

At least **10 putative epitopes** on the capsid protein, located on the external VP60 loops, were preliminary mapped

Conclusions

- RHDV2 is present as quasi-species and different strains, also originating from events of recombination, are circulating in Italy and presumably also in other countries
- A continuous surveillance during RHD outbreaks is needed to trace the evolutionary steps of this virus
- The RHDV2 broad genetic variability should be verified in terms of consequent changes of its antigenic properties
- The approach of merging the VP60 aminoacidic sequences of several strains with their antigenic profiles, by mapping the epitopes exposed on the surface of the virus, will permit to find the “best strain” to develop even more effective vaccines

ACKNOWLEDGEMENTS

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

Part of this data will be included in the EU PRIMA-LAGMED project *Improvement of preventive actions to emerging **LAG**oviruses in the **MED**iterranean basin: development and optimisation of methodologies for pathogen detection and control*