



# PROCEEDINGS OF THE 11<sup>th</sup> WORLD RABBIT CONGRESS

Qingdao (China) - June 15-18, 2016

ISSN 2308-1910

## Session Pathology and Hygiene

**Marino M., Pugliese N., Circella E., Cocciolo G., Papapicco C.,  
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### Full text of the communication

*How to cite this paper :*

*Marino M., Pugliese N., Circella E., Cocciolo G., Papapicco C., Tondo A., Romito D., D'Onghia F., Camarda A., 2016 - Circulation of different strains of rabbit hemorrhagic disease virus (RHDV) in southern Italy: clinical and epidemiological findings. Proceedings 11th World Rabbit Congress - June 15-18, 2016 - Qingdao - China, 571-574.*



## CIRCULATION OF DIFFERENT STRAINS OF RABBIT HEMORRHAGIC DISEASE VIRUS (RHDV) IN SOUTHERN ITALY: CLINICAL AND EPIDEMIOLOGICAL FINDINGS

Marino M.<sup>1</sup>, Pugliese N.<sup>1</sup>, Circella E.<sup>1</sup>, Cocciolo G.<sup>1</sup>, Papapicco C.<sup>1</sup>, Tondo A.<sup>1</sup>, Romito D.<sup>1</sup>, D'Onghia F.<sup>1</sup>, Camarda A.<sup>1\*</sup>

<sup>1</sup>Department. of Veterinary Medicine, University of Bari "Aldo Moro",  
S.P. per Casamassima Km 3, 70010, Valenzano (Bari), Italy  
\*Corresponding author: antonio.camarda@uniba.it

### ABSTRACT

The Rabbit Hemorrhagic Disease Virus (RHDV) belongs to the genus *Lagovirus* (Family: Caliciviridae) and causes rabbit hemorrhagic disease (RHD) in rabbits (*Oryctolagus cuniculus*). In 2010, a new RHDV-related virus (RHDV2) with a unique genetic and antigenic profile was identified in France in rabbits. This new virus has been quickly spreading in many parts of the world. In Italy, RHDV2 has been reported in wild and domestic animals from the entire Country. This study was aimed to describe 13 RHD cases, which occurred in Southern Italy, where several outbreaks occurred in a short time. We detected RHDV2 in most of cases, but some RHD cases were due to the "classic" RHDV or to the RHDVa variant. The evidenced wide diffusion of RHDV2 in Southern Italy is cause of concern as RHD is a serious problem for farmers due to the induced severe losses. Considering the high infectious potential of the virus, it is an absolute necessity the implementation of control measures, among those the use of specific homologues vaccines, to reduce the risk of diffusion and new outbreaks.

**Key words:** Rabbit Hemorrhagic Disease Virus (RHDV), European rabbit (*Oryctolagus cuniculus*), RHDV2, RT-PCR, diagnosis, epidemiology

### INTRODUCTION

Rabbit hemorrhagic disease (RHD) is a fatal and highly contagious infectious disease that affects wild and domestic European rabbits (OIE, 2015). The disease is characterized by 100% morbidity and high mortality rates (>70%). Gross lesions include hyperemia, congestion and diffuse hemorrhages, liver degeneration, necrosis and splenomegaly (Marcato et al., 1991).

The etiological agent of RHD is the rabbit haemorrhagic disease virus (RHDV), a non-enveloped virus with a positive-sense, single-stranded RNA genome (Capucci et al., 1990; Meyers et al., 1991b), belonging to the family Caliciviridae, genus *Lagovirus*. The genus includes, among others, the European brown hare syndrome virus (EBHSV), etiological agent of the European brown hare syndrome (EBHS) a similar disease affecting affects brown, mountain and Italian hare (Frolich et Lavazza, 2007). Such virus is closely related to RHDV, with very similar morphology, but it is well antigenically and genetically distinct (Capucci et al., 1991; Poli et al., 1991, Chasey et al., 1992).

To date, RHDV strains have been genetically divided into six genogroups, denoted G1-G6, and clustered according to the year of isolation and not according to their geographic location (Le Gall-Reculé et al., 2003). The cluster G6 represents an exception, as the included strains showed no geographic or temporal links among

themselves. Instead, it consisted of a group of strains, which differed from the other RHDVs for their antigenic profile, and it was named RHDVa (Capucci et al., 1998).

In 2010 a new RHDV related virus (termed RHDV2) was identified in France firstly in wild rabbits and then in domestic farmed rabbits. It was characterized by a genetic and antigenic profile clearly different from RHDV. The data suggest that RHDV2 is not a variant of RHDV but a new emerging virus (Le Gall-Reculé et al., 2013) and it is nowadays spread and reported in most European countries Europe. In Italy this new virus, which could be considered like a new serotype, was firstly identified in rabbits on 2011 (Le Gall et al., 2013) but it has also been identified in the Sardinian Cape hare (Puggioni et al., 2013) and Italian hare (Camarda et al., 2014)

The aim of the our study was to evaluate the spread of RHDV2 during a three years period (2013-2015) in geographic locations of South Italy and to genetically characterize RHDV strains obtained from rabbits in areas of South Italy.

## MATERIALS AND METHODS

### **Rabbit hemorrhagic disease foci.**

Between March 2013 and May 2015 we investigated 12 intensive and 6 rural rabbit farms in which medium-high mortality (over 20%) was recorded. All farms were located in Southern Italy. Specifically twelve were in Apulia, three in Basilicata, two in Sicily and one in Calabria .

Liver samples were collected from dead rabbits from intensive or rural rabbit farms in the South Italy. Upon arrival to the facilities of the Department of Veterinary Medicine, University of Bari (Italy), 3-6 animals were necropsied and samples of liver were collected. Total RNA was extracted from livers by using the RNeasy Mini Kit (Qiagen, Milan, Italy) according to the manufacturer's instructions. A RT-PCR, targeting the gene encoding the VP60 structural capsid protein with an expected amplicon of about 560 bp, was carried out as previously described (Camarda et al., 2014).

PCR products were separated by gel electrophoresis in 1.5% agarose gel followed by staining with ethidium bromide 0.5 µg/mL. Stained agarose gels were exposed to UV and image were digitalized by mean of a GelDoc-It Imaging System (UVP, Upland, CA, US).

The gathered amplicons were cloned in pGEM-T Easy cloning vector (Promega, Milan, Italy) according to the manufacturer's protocol, and sequenced by the Big Dye Terminator v3.1. Sequence determination was performed on an Applied Biosystem ABI 3100 at the facilities of BMR Genomics (Padova, Italy). The identification of the products was carried out by comparing the nucleotide sequences with those in GenBank through BLAST.

## RESULTS AND DISCUSSION

Out of the 18 investigated cases, 9 intensive and 4 rural rabbit farms were confirmed as RHD foci, as the RT-PCR returned the expected band of about 560 bp, and the BLAST analysis of the sequence of amplicons confirmed the presence of RHDV. The sequence also allowed the classification of viruses as RHDV, RHDVa and RHDV2. Out of the 9 cases from Apulia, 6 were caused by RHDV2, 2 by RHDVa and only one by RHDV. RHDV2 was also detected from all samples in the two foci in Sicily and the one in Calabria, while the strain from Basilicata was RHDVa (table 1).

The RT-PCRs confirmed the evidence from clinical and analytical investigations. In fact, the RHD foci were characterized by the observation of clinical signs of RHDV. In addition, the typical lesions of RHD were observed in all the examined dead rabbits. At necropsy, disseminated haemorrhages, blood foam in the lumen of trachea, hepato- and splenomegaly were evident. Additionally, livers were sensibly swollen. However, mortality rate was greatly variable among the foci. In particular, mortality due to RHDV2 varied from about 25-30% to 90-100%.

**Table 1:** Details about the described RHD foci

N°	Type	Origin	Region	Year	RHDV Strain
1	intensive	Foggia	Apulia	2013	RHDV2
2	rural	Catania	Sicily	2013	RHDV2
3	intensive	Laurenzana (PZ)	Basilicata	2013	RHDVa
4	intensive	Caltanissetta	Sicily	2013	RHDV2
5	rural	Gioia del Colle (BA)	Apulia	2014	RHDV
6	intensive	Gioia del Colle (BA)	Apulia	2014	RHDV2
7	rural	Ostuni (BR)	Apulia	2014	RHDV2
8	intensive	Modugno (BA)	Apulia	2014	RHDV2
9	intensive	Maritina Franca (TA)	Apulia	2014	RHDV2
10	intensive	Vibo Valentia	Calabria	2014	RHDV2
11	intensive	Foggia	Apulia	2014	RHDVa
12	rural	Foggia	Apulia	2014	RHDVa
13	intensive	Santeramo (BA)	Apulia	2015	RHDV2

We found an association between the mortality rate and the vaccination history of the animals. Wherever vaccination was carried out (with vaccines derived from RHDV and RHDVa), mortality was lower, while, in the farms where no vaccination was carried out, up to 100% of rabbits died. Data are not enough to perform a statistically significant analysis of such findings, and other factors (i.e. the pathogenicity of the strains and the serological response of the animals) should be taken into account, but this different behavior may let hypothesize that RHDV-derived vaccines offer low but not negligible protection, maybe due to some cross-reactivity between the RHDV and RHDV2 antigens.

The presented data show that RHDV2 has had a wide spread in Southern Italy. In the last two years, the majority of RHDV foci was due to the new variant. The first foci of RHDV2 in Italy were reported from the Northern regions starting from mid-2011, but thereafter RHDV2 the diffusion of this virus in the whole country, including main islands, occurred very quickly with scattered outbreaks often without any epidemiological link. However, despite the rapid circulation of such new virus, it did not completely overcome the diffusion of “classical” RHDV strains, as four 4 of the 13 analyzed foci were caused by RHDV or RHDVa. Interestingly, there is no association between the kind of farm (rural or intensive), and the RHDV strain. This makes difficult to hypothesize the possible routes of transmission.

The transmission of the disease may occur through different routes: direct contact with an infected animal (Patton 1989); indirectly by contaminated food, water, bedding, cages or equipment, vehicles and operators (Cooke 2002); through the wild animals which predates infected animals or their carcasses and then release the virus through the feces (Frölich et al 1998); and in a vector-borne fashion (Cooke, 2002).

The resistance of virus to the environmental conditions facilitates its diffusion. In fact, the persistence of the virus in the rabbit carcasses is at least three months and this makes wild rabbits a reservoir of virus (Henning et al., 2005).

The diffusion of RHDV2 in the wild and natural environment may be aided by its lower mortality, which could allow a broader dislocation of the infected animals.

## CONCLUSIONS

According to the collected data, the RHD is spreading rapidly in the rabbit farms of Southern Italy. This is a serious problem for the rabbit’s health but also causes significant economic losses for farmers.

The data collected in Southern Italy, although partial, indicate that the virus moves quickly. Given its high infectivity, without prompt actions and accurate vaccinations, the risk of new and widespread outbreaks, remains very high. Therefore, it would be advisable to make available new and effective vaccine towards RHDV2 all over the Europe,

especially in the endemic RHD areas. It would be also important to use vaccines based on strains showing a high level of homology with field strains (e.g. autovaccines) in order to induce high level of immunity and to avoid the selection of more virulent strains as result of selective pressure. Furthermore, it should be taken into account the possibility to implement a capillary vaccination campaign which involves both industrial and rural rabbit farms. In fact, the latter contribute to the circulation of RHDV and RHDV2, and they may represent a reservoir of infection.

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