

PROGRESSION OF RABBIT HAEMORRHAGIC DISEASE VIRUS 2 UPON VACCINATION IN AN INDUSTRIAL RABBITRY: A LABORATORIAL APPROACH

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Abstract: Rabbit haemorrhagic disease virus 2 (RHDV2) emerged recently in several European countries, leading to extensive economic losses in the industry. In response to this new infection, specific inactivated vaccines were developed in Europe and full and rapid setup of protective immunity induced by vaccination was reported. However, data on the efficacy of these vaccines in an ongoing-infection scenario is unavailable. In this study we investigated an infected RHDV2 indoor industrial meat rabbitry, where fatalities continued to occur after the implementation of the RHDV2 vaccination, introduced to control the disease. The aim of this study was to understand if these mortalities were RHDV2-related, to discover if the dead animals showed any common features such as age or time distance from vaccination, and to identify the source of the outbreak. Anatomico-pathological analysis of vaccinated animals with the virus showed lesions compatible with systemic haemorrhagic disease and RHDV2-RNA was detected in 85.7% of the animals tested. Sequencing of the *vp60* gene amplified from liver samples led to the recognition of RHDV2 field strains demonstrating that after the implementation of vaccination, RHDV2 continued to circulate in the premises and to cause sporadic deaths. A nearby, semi-intensive, RHDV2 infected farm belonging to the same owner was identified as the most probable source of the virus. The main risk factors for virus introduction in these two industries were identified. Despite the virus being able to infect a few of the vaccinated rabbits, the significant decrease in mortality rate observed in vaccinated adult rabbits clearly reflects the efficacy of the vaccination. Nonetheless, the time taken to control the infection also highlights the importance of RHDV2 vaccination prior to the first contact with the virus, highly recommendable in endemic areas, to mitigate the infection's impact on the industry.

Key Words: rabbit, RHDV2, rabbit haemorrhagic disease, vaccines.

INTRODUCTION

Both rabbit haemorrhagic disease virus 2 (RHDV2) and rabbit haemorrhagic disease virus (RHDV) are classified within the *Lagovirus* genus along with the genetically related European brown hare syndrome virus (EBHSV)(Capucci *et al.*, 1991; Le Gall-Reculé *et al.*, 2013). RHDV2 was first reported in 2010 (Le Gall-Reculé *et al.*, 2011), whereas classic

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RHDV strains have circulated worldwide since 1984 (Nowotny *et al.*, 1997; Capucci *et al.*, 1998; Le Gall *et al.*, 1998; Le Gall-Reculé *et al.*, 2003).

Although rabbit haemorrhagic disease (RHD) was first described in China during the 1980s in Angora rabbits that had been imported from Germany, the virus is thought to have originated in Europe (Liu *et al.*, 1984). It causes a lethal and contagious disease which presents with liver necrosis, splenomegaly and haemorrhagic lesions in the liver and in the lungs (Capucci *et al.*, 1991; Marcato *et al.*, 1991). Typically, diseased animals present with fever (>40°C), and death occurs rapidly within 12 to 36 h after exposure (Capucci *et al.*, 1991; Marcato *et al.*, 1991; McIntosh *et al.*, 2007). Common clinical signs include a blood-tinged foamy nasal discharge, severe respiratory distress and/or convulsions, usually preceding death (Marcato *et al.*, 1991; McIntosh *et al.*, 2007). Mortality rates are high, ranging from 80% to 100% (Marcato *et al.*, 1991; Ohlinger *et al.*, 1993).

RHDV2, also known as RHDVb, is closely related to RHDV but also highly genetically distinctive and therefore represents a new genotype (Le Gall-Reculé *et al.*, 2011, 2013). After its emergence in France (Le Gall-Reculé *et al.*, 2011), it quickly spread to other European countries (revised in Duarte *et al.*, 2015a), replacing the classic strains previously circulating in France (Le Gall-Reculé *et al.*, 2013), the Iberian Peninsula (Bárcena *et al.*, 2015; Calvete *et al.*, 2014; Dalton *et al.*, 2014; Lopes *et al.*, 2015) and the Azores (Duarte *et al.*, 2015a). RHDV2 was also reported in an isolated case in Australia (Hall *et al.*, 2015). In addition to the European rabbit, RHDV2 is able to infect a few hare species (Puggioni *et al.*, 2013; Camarda *et al.*, 2014).

RHDV2 is less virulent than RHDV, and therefore associated with lower mortality rates (Le Gall-Reculé *et al.*, 2013). The disease developed also differs from that induced by RHDV in its clinical presentation, given that chronic and sub-acute forms are more frequent in RHDV2 infections (Le Gall-Reculé *et al.*, 2013) whereas in RHDV infections only a small percentage of animals (5 to 10%) develop a sub-acute or chronic illness presenting with jaundice, malaise, weight-loss and death within 1 to 2 wk after the onset of symptoms (McIntosh *et al.*, 2007).

Nestlings as young as 11 d old are susceptible to RHDV2 and develop the disease (Dalton *et al.*, 2012), unlike in the case of RHDV, to which kits up to 4 wk of age are naturally resistant (Liu *et al.*, 1984). Regardless of age-independent susceptibility, the lack of cross protection induced by previous contact with RHDV strains contributed to the rapid spread of RHDV2 in Europe (Le Gall-Reculé *et al.*, 2013), resulting in high mortality rates among wild populations soon after its emergence.

In the industry, RHDV2 was reported for the first time in France (Le Gall-Reculé *et al.*, 2011), and soon after in Great Britain (Baily *et al.*, 2014; Westcott *et al.*, 2014) and the Iberian Peninsula (Dalton *et al.*, 2012; Duarte *et al.*, unpublished results), resulting in severe losses in this sector. RHDV-vaccinated rabbits are totally protected against RHDV infection but only partially to RHDV2. Hence, in farms with no RHDV vaccination in place the mortality rates induced by RHDV2 rose to 80%, while in vaccinated animals the rate observed was considerably lower (25%) (Le Gall-Reculé *et al.*, 2013). Still, the limited cross protection against RHDV2 conferred by inactivated or recombinant RHDV vaccines (Torres *et al.*, 2000; Le Gall-Reculé *et al.*, 2011, 2013; Dalton *et al.*, 2014) led to the development of specific RHDV2 inactivated vaccines in Europe (Filavac VHD Variant, Filavie Laboratories, Cunipravic RHD variant, Hipra; Novarvilpac, Ovejero). These vaccines were provisionally allowed in the European Union member states, as their use requires special licenses from the Local Veterinarian Authorities, and vaccination against RHDV2 has become common practice in the industry.

The full and rapid setup of RHDV2 protective immunity induced by Filavac VHD Variant vaccine, established within one week, was reported in four- and 10-wk old rabbits (Minor *et al.*, 2013). No cross protection against the classic virus was observed (Le Minor *et al.*, 2013). However, there is no data available on the use of vaccination in an ongoing-RHDV2 infection scenario.

In this study, we investigated a series of fatalities that occurred in an indoor industrial meat rabbitry after the implementation of the RHDV2 vaccination. To clarify the cause of the fatalities, we gathered and integrated clinical, epidemiologic, anatomo-histopathologic, virologic and bacteriologic data. After the identification and molecular characterisation of RHDV2 as the etiological agent, the research focused on detection of the probable infection source, transmission routes and the identification of possible reasons for infection recurrence after vaccination. Our data allow preliminary conclusions on the efficacy of vaccination as a therapeutic measure.

MATERIALS AND METHODS

Sample origin and epidemiological inquiry

A total of twenty-one dead rabbits were analysed. Nine rabbits had not been vaccinated and 12 were vaccinated once or twice against RHDV2. Fourteen of these 21 animals originated from the indoor, semi-intensive, meat rabbitry investigated in this study, located in the north of Portugal, hereby referred to as *cv-Farm* due to its controlled ventilation system. The other 7 samples originated from a second industrial farm, located 300 m away, referred to as *nv-Farm*, given its natural ventilation. The casualties occurred between January 2015 and August 2015 (Table 1). The cadavers or organs were received at a private laboratory, Segalab, S.A., and then sent to the Instituto Nacional de Investigação Agrária e Veterinária (INIAV).

An inquiry was carried out to gather information at the *cv-Farm* facilities, its operating system, production rates, reproductive strategies, prophylactic measures, and, regarding the 21 specimens' age, immunisation profile and vaccination date, time of death, and necropsy data (if performed at the rabbitry). Information on the overall mortality among adults and kits was also collected. Cases were numbered according to their chronological occurrence.

Anatomo-histopathological examination

Necropsies were performed by the veterinarian assistant at the rabbitry or by the pathologists at the Pathology Laboratory, INIAV.

For anatomo-pathological examinations, liver and lung samples were fixed in 10% buffered formalin and embedded in paraffin using standard procedures. Five micrometre-thick sections were stained with haematoxylin and eosin (H&E) and examined using light microscopy (Cook, 1997).

Bacteriological analysis

Liver and lung samples from the 21 animals were analysed using standard bacteriological methods, and the presence of *Pasteurella* sp., which is to be considered in the differential diagnosis of RHD according to the OIE (World Organisation for Animal Health (OIE) Technical disease cards, 2015), was investigated. Lung and liver sample macerates were inoculated in MacConkey agar (Oxoid) and Colombia agar (Oxoid), supplemented with 5% of defibrinated sheep blood (Biomérieux) and incubated at 37°C for 24-48 h. Identification of isolates was performed using the commercial API® test strips API 20 NE and API ID32 E (BioMérieux). To infer the sanitary status of the animals, which is a public health concern as they are used for human consumption, the presence of verocytotoxin (VT) producing *Escherichia coli* strains (VTEC) was investigated by multiplex PCR (Paton and Paton, 1998). *E. coli* strains ED647 (*E. coli* 0157, *vt1*, *vt2*, *eae*) and ED378 (*O18ab*, *vt2f*), provided by the European Reference Laboratory for VTEC, Instituto Superiore di Sanità Italy, were used as positive controls and *E. coli* strain JM109 as a negative control.

Virological examination

Tissue samples comprising liver and lungs were homogenised with phosphate buffered saline (PBS) and clarified at 3000 *g* for 5 min. DNA and RNA were extracted from 200 µL of the clarified supernatant, corresponding to approximately 50 mg of tissue, in a BioSprint 96 nucleic acid extractor (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

With regard to vaccines, RNA from vaccine Cunipravac RHD variant (Hipra, Spain) was extracted from the aqueous phase of a centrifuged sample (10000 *g* for 10 min), using the RNeasy blood and tissue kit (Qiagen, Hilden, Germany) according to the recommendations. RNA from vaccine Novarvilap (Ovejero, Spain) was extracted from 200 µL of a 10× diluted sample (*v/v* in bidistilled H₂O), in a BioSprint 96 nucleic acid extractor (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

Samples were tested for RHDV2 by RT-qPCR (Duarte *et al.*, 2015b). Screening for RHDV was performed by sequencing analysis of the amplicons obtained by conventional PCR with primers RC-9 and RC-10 (Tham *et al.*, 1999). Conventional RT-PCR and RT-qPCR were performed using the One Step RT-PCR kit (Qiagen, Hilden, Germany).

Table 1: Information on the cases investigated; age at the time of death, vaccination history, virological laboratorial results and accession numbers of vp60 gene sequences.

Case No.	Date of death	Age group	GenBank accession # <i>vp60</i>	Farm of origin	MYXV Mixohipra	RHDV Anvilap	Vaccinations		Vaccination date for RHDV2	Time gap (d) between last vaccination and death	Virological examination		
							RHDV Anvilap	RHDV2 vaccine Novarvilap (1) Cunitpravac (2)			RHDV2 (Cq)	RHDV	MYXV
0	22 Oct14	Adult	KU665601 ^c	nv-Farm	Y	Y	-	-	Not vaccinated	-	12.58	N	N
1	28 Oct14	Adult	KU665600 ^c	cv-Farm	Y	Y	-	-	Not vaccinated	-	11.83	N	N
2	06 Jan15	Adult	ND	cv-Farm	Y	Y	1	30 Oct14	30 Oct14	68	31.71	N	N
3	06 Jan15	Adult	KU665598 ^c	cv-Farm	Y	Y	1	30 Oct14	30 Oct14	68	9.53	N	N
4	03 Feb15	Adult	-	cv-Farm	Y	Y	1	30 Oct14	30 Oct14	96	no Cq	N	N
5	28 Feb15	Adult	ND	cv-Farm	Y	Y	1	30 Oct14	30 Oct14	121	33.09	N	N
6	28 Feb15	Adult	ND	cv-Farm	Y	Y	1	30 Oct14	30 Oct14	121	32.17	N	N
7	17 Mar15	Adult*	KU665597 ^c	cv-Farm	Y	Y	1,2	30 Oct14, 02 Mar15	138, 15	138, 15	15.27	N	N
8	17 Mar15	Adult*	-	cv-Farm	Y	Y	1,2	30 Oct14, 02 Mar15	138, 15	138, 15	29.08	N	N
9	17 Mar15	Young (35d)	ND	cv-Farm	Not vaccinated	Not vaccinated	2	02 Mar15	15	15	16.85	N	N
10	17 Mar15	Young (35d)	KU665599 ^b	cv-Farm	Not vaccinated	Not vaccinated	2	02 Mar15	15	15	11.82	N	N
11	17 Mar15	Young (35d)	ND	cv-Farm	Not vaccinated	Not vaccinated	2	02 Mar15	15	15	12.74	N	N
12	29 Mar15	Adult	ND	cv-Farm	Y	Y	-	Not vaccinated	-	-	11.56	N	N
13	29 Mar15	Adult	ND	cv-Farm	Y	Y	1	02 Mar15	27	27	36.14	N	N
14	20 Apr15	Adult	-	cv-Farm	Y	Y	2	02 Mar15	48	48	no Cq	N	N
15	22 Apr15	Young (>35d)	KU665594 ^c	nv-Farm	Not vaccinated	Not vaccinated	-	Not vaccinated	-	-	16.22	N	N
16	22 Apr15	Young (>35d)	KU665596 ^c	nv-Farm	Not vaccinated	Not vaccinated	-	Not vaccinated	-	-	17.29	N	N
17	22 Apr15	Young (>35d)	KU665595 ^c	nv-Farm	Not vaccinated	Not vaccinated	-	Not vaccinated	-	-	22.46	N	N
18	24 Jul15	Young (59d)	ND	nv-Farm	Not vaccinated	Not vaccinated	-	Not vaccinated	-	-	21.60	N	N
19	24 Jul15	Young (59d)	ND	nv-Farm	Not vaccinated	Not vaccinated	-	Not vaccinated	-	-	20.10	N	N
20	18 Aug15	Young (>35d)	-	nv-Farm	Not vaccinated	Not vaccinated	-	Not vaccinated	-	-	no Cq	N	N

ND: Not determined; *Multiparous doe, that had given birth 11 d prior; +: Multiparous doe, in the initial stage of pregnancy; Y: Yes; N: Negative; cv-Farm: controlled ventilated Farm; nv Farm: natural ventilated Farm; c: Complete; p: partial

The presence of myxoma virus was examined by qPCR (Duarte *et al.*, 2014), using the FastStart TaqMan Probe Master Kit (Roche, Roche Diagnostics GmbH, Mannheim, Germany).

C_q (quantification cycle) values are inversely related to the concentration of nucleic acid in the sample (revised by (Gullett and Nolte, 2015)). For the real time PCR systems described, undetectable C_q or C_q values >40 were considered negative.

Nucleotide sequencing analysis

Amplification of the *vp60* sequences of RHDV2 strains was accomplished with 2 pairs of primers, 27F (5'-CCATGCCAGACTTGGCGTCCC-3') and 986R (5'-AACCATCTGGAGCAATTTGGG-3'), 717F (5'-CGCAGATCTCCTCACAACCC-3') (Duarte *et al.*, 2015a), and RC10R (Tham *et al.*, 1999) enabling us to obtain 2 overlapping fragments. The One Step (Qiagen, Hilden, Germany) kit was used, following the manufacturers' recommendations. Sequencing was accomplished using the BigDye™ Terminator cycle sequencing kit (Applied Biosystems, Foster City, CA, USA).

The *vp60* nucleotide sequences of 7 complete and 1 partial RHDV2 strains (GenBank accession numbers KU665594 to KU665601) were determined in an automated 3130 Genetic Analyzer system (Applied Biosystems, Foster City, CA, USA).

Nucleotide diversity (π), and its corresponding variances (Nei, 1987) were estimated independently for each sub-population (nv-Farm and cv-Farm strains) as well as for both sub-populations combined, resorting to DNASP software (Version 5.10.01) (Rozas *et al.*, 2003). Standard errors (SE) of each measure were based on 1000 bootstrap replicates. DnaSP was also used to calculate the minimum number of recombination events (R_m) in the sample (Hudson and Kaplan, 1985).

RESULTS

Insights from the epidemiologic inquiry

The inquiry revealed that the animals originated from a high-standard rabbitry (cv-Farm) holding 800 does which produce around 6000 animals per productive cycle. Does, weighing between 3.5 and 4.5 kg, give birth every 42 d as artificial insemination is practiced 11 d postpartum, producing an average of 22 kg of meat per inseminated doe. The cv-Farm comprises separate areas for artificial breeding and fattening, and works in a closed, all-in-all-out system, with controlled temperature (22 to 26°C), humidity, artificial light and ventilation. Kits are weaned at 32 d of age, at which point the mothers are moved to a contiguous area where the next productive cycle begins. Fattened rabbits are collected for slaughtering at 70 d of age. Facilities are disinfected by a specialised company and kept empty for a week before the following breeding cycle. We also found out that a second semi-intensive farm (nv-Farm), belonging to the same owner, is located 300 metres away from the cv-Farm. Both farms are sited on agricultural land. No movement of animals was carried out between farms, but sharing of workers was identified.

Epidemiological data linking the farms is schematised in Figure 1.

Regarding the prophylactic measures in practice prior to RHDV2 introduction, it was disclosed that disease control was carried out by the veterinary assistant according to an established programme. RHDV vaccination (Arvilap) was carried out twice a year (spring and autumn), and Myxoma virus vaccination (MYXV) (Mixohipra H, Hipra) every 4.5 mo. Control of internal parasites is achieved by administering albendazole, levamisole, or fenbendazole, alternately, every other productive cycle. Insecticides are regularly used for mosquito control. Other preventive sanitary prophylactic measures include rodent and plague control by a certified company, which periodically monitors the installed devices (such as bait boxes with raticide). Ventilators are sited far from the communication doors and thus from any contact with the slaughter and food vehicles as well as the entries through which the animals enter and exit. In addition, cadavers are collected 2 km away from the farm by a specialist company, so that this vehicle does not approach the farm's surrounding area. There are no specific nets to avoid contacts with wild rabbits. All the farm workers use specific equipment and visitors are not allowed.

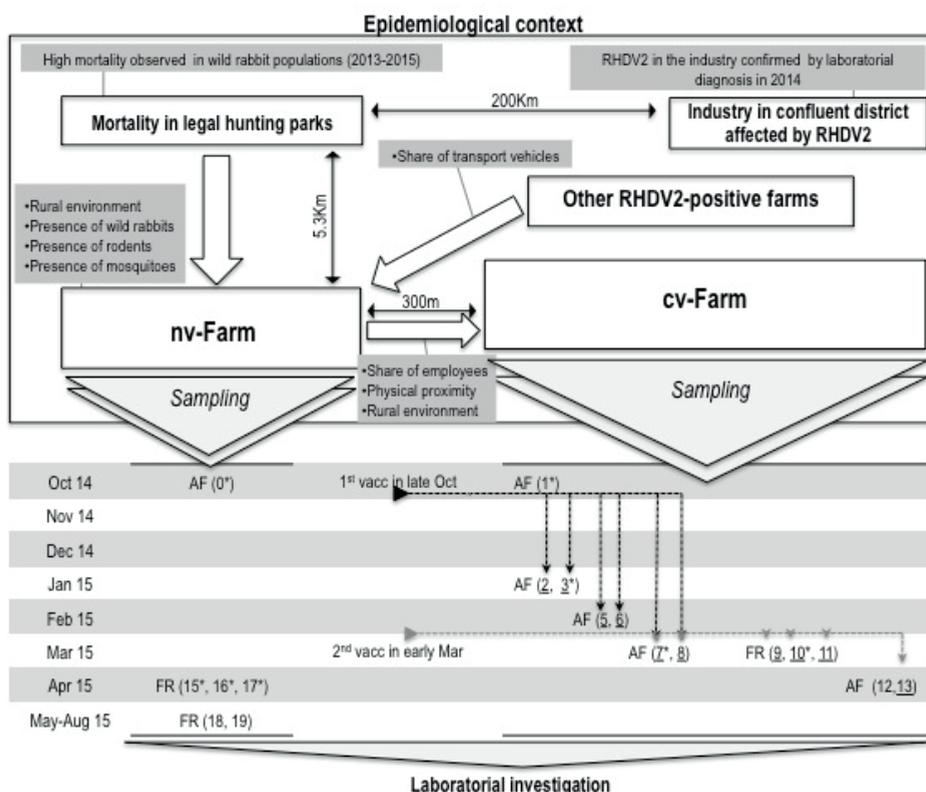


Figure 1: Top, schematic representation of the epidemiological context of the farm. Bottom, information on the RHDV2-positive sampling with regards to farm of origin, date of collection and vaccination. Black arrows represent geographical distances (not in scale). White arrows represent the possible origin and dissemination paths. Risk factors are shown within grey boxes. Numbers in brackets refer to case numbers, which position in relation to the calendar (on the left) refers to time of death. The case numbers corresponding to vaccinated rabbits are underscored. AF, adult females; FR, fattening rabbits; nv-Farm, natural ventilated farm; cv-Farm, controlled ventilated farm. Vaccines are represented by triangles (black, Novarvilap; grey, Cunipravec RHD variant). *Strains with *vp60* gene sequenced.

The events started in late October 2014, when a series of deaths were registered in the cv-Farm, with mortalities of 80% in juveniles and 30-40% in does (Case 1, adult female), respectively. The enquiry revealed that a week earlier, several animals of all ages had begun to die in the nv-Farm (Case 0, adult female). RHDV2 presence was confirmed in both farms by laboratory diagnosis and, RHDV2 vaccination of the adult females was implemented with Novarvilap (Ovejero Laboratories).

Two months later, in January 2015, 2 pregnant females from the cv-Farm, which had been vaccinated 68 d earlier, died suddenly without any clinical signs of disease (cases 2 and 3, Table 1). Macroscopic examination of these rabbits revealed good body condition, but haemorrhages in the lungs, icteric liver and splenomegaly, raising the suspicion of RHD. RHDV2 was confirmed by laboratorial diagnosis. A second RHDV2 outbreak was observed in the cv-farm in late February to early March. Newly weaned 27 d-old kits and 35 and 60 d-old rabbits were affected, most of which were offspring of primiparous females vaccinated once, 121 d prior. Adults also fell victim and lesions compatible with haemorrhagic disease were observed in a few females vaccinated 121 d earlier (cases 5 and 6, Table 1).

Limitations from the supplier led to the introduction of a second RHDV2 vaccine (Cunipravic RHD variant, Hipra, Spain) in both farms at the beginning of March and the enquiry revealed that a few weaned kits were also vaccinated once with this vaccine.

The last mortalities in the cv-Farm occurred in mid-late March, affecting vaccinated females (cases 7, 8, 13, Table 1), including one multiparous that had given birth recently (case 7) and another pregnant multiparous (case 8). Females 7 and 8 had been vaccinated twice, with the Norvavilap and Cunipravic RHD variant, 138 and 15 d before, respectively. Female 13 was vaccinated once with the Cunipravic RHD variant, 27 d before. Thirty-five-day old kits, vaccinated once with the Cunipravic RHD variant (cases 9, 10, 11, Table 1), also fell victim.

In the nv-Farm, mortalities due to RHDV2 continued to occur until late July 2015, affecting non-vaccinated fattening kits aged 35 to 70 d (cases 15-19, Table 1). No more casualties were observed thereafter.

Microbiology

RHDV2 was detected in 18 of the 21 rabbits analysed (Cases 0-3, 5-13, 15-19, Table 1, and Figure 2), by RT-qPCR (Duarte *et al.*, 2015b). Viral loads were variable, as Cq values ranged from 9.53 to 36.14, with high viral charges (Cq<25) found in the majority of the samples tested (n=13/18, 72.22%). Despite the limited sampling, a positive association was observed between viral loads and lesion severity in kits (Figure 2).

Only 3 out of the 21 animals analysed were negative for RHDV2 (cases 4, 14 and 20, Table 1). Data of the 18 RHDV2-positive cases, regarding sample origin, collection and vaccination date, are represented in Figure 1 (bottom).

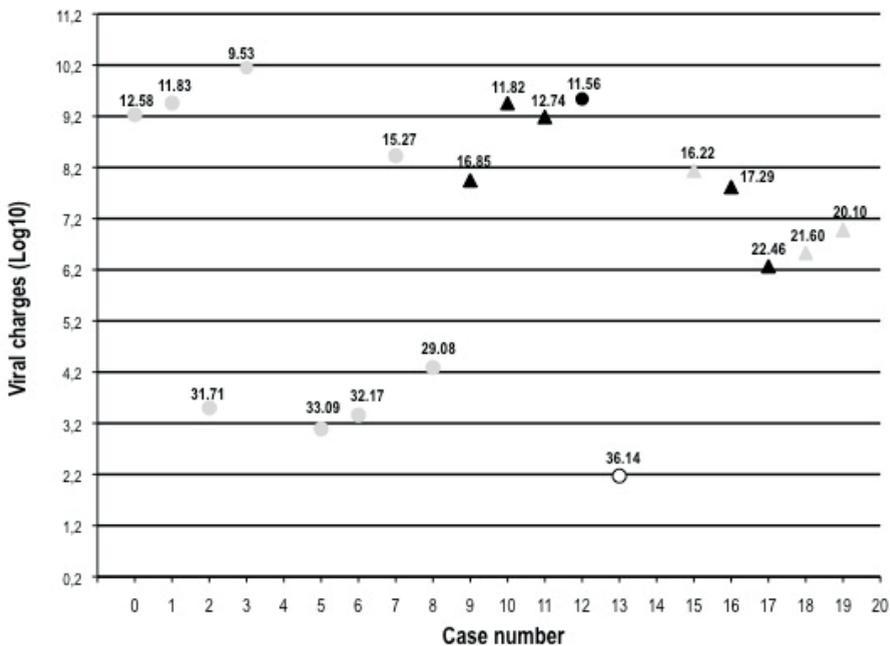


Figure 2: Viral charges found in RHDV2-positive samples. Circles represent adults and triangles the young. Colour corresponds to samples where microscopic lesions were observed (black), not determined (grey) or absent (white). Viral charges were inferred from the respective Cq values according to the regression equation published by Duarte *et al.*, 2015b. Cq values are shown above the symbols. Cases 4, 14 and 20 (RHDV2-negative) are not represented.

Pasteurella multocida was isolated from the lungs of one RHDV2-negative multiparous doe (case 4). Neither *Salmonella*, *Yersinia*, *Staphylococcus* nor *Listeria* were detected. *E. coli* was isolated from the tissues of 3 adult females (cases 5, 6 and 7) and 3 kits (cases 9, 10 and 11), all RHDV2-positive rabbits, but no associated virulence genes (*vtx1*, *vtx2*, *eae* and *vtx2f*) were found.

Anatomo-histopathology

No signs of disease were registered prior to death in any of the 21 rabbits. Bloody discharges from the nose, observed in 5 animals (23.81%, case 4 [RHDV2-negative], and cases 7, 9, 10 and 11, all RHDV2-positive), were the only outward manifestation of an ongoing pathology.

Macroscopically, in the RHDV2 positive animals, an icteric liver and hepatomegaly were the most common lesions (90 to 100%) followed by lung petechiae (45 to 50%) and moderate splenomegaly (30%). Hepatic discoloration was also observed (cases 9, 10 and 11, all referring to RHDV2 positive kits).

At microscopic level, the great majority of the lesions in the RHDV2-positive rabbits matched the typical RHD lesions described above (Ohlinger *et al.*, 1993). Those included microfoci of hepatic necrosis, detected in the liver parenchyma of 6 animals (cases 9-12, 16 and 17, all RHDV2-positive; cases 9-11 and 17 vaccinated, and cases 12 and 16 not vaccinated). Hepatocyte hyalinisation was found in 5 of these samples (cases 9-11, 16 and 17). Lung histopathology was performed in 2 specimens showing severe congestion and disseminated intravascular coagulation in the small capillaries (cases 9 and 10).

In the adult female case 4, lesions consisted of purulent pneumonia with extensive parenchymal infiltration by inflammatory cells, mainly neutrophils, and occasional bacteria clumps associated with necrotic foci.

Despite some degree of autolysis, no lesions were recognised in the liver or lungs of the adult rabbit case 13, where a RHDV2 high Cq value (36.14) was obtained.

Insights from the molecular analysis

Similarity among field strains was above 99.43%, with 2 of the complete sequences being identical (KU665597 and KU665598, Table 2) and 2 differing only in 1 nucleotide (KU665595 and KU665596, Table 2). The average number of nucleotide differences (k) and the nucleotide diversity (π) found among all *vp60* sequences showed intermediate

Table 2: Nucleotide similarity percentage among the *vp60* nucleotide sequences of two vaccines and eight field RHDV2 strains.

Strains	Case 0	Case 1	Case 3	Case 7	Case 10*	Case 15	Case 16	Case 17	Vaccine A	Vaccine B
Case 0#	-	100	99.60	99.83	99.90*	99.89	99.83	99.83	98.74	97.82
Case 1†		-	99.60	99.83	99.90*	99.89	99.83	99.83	98.74	97.82
Case 3†			-	99.43	99.18*	99.48	99.54	99.54	98.91	97.76
Case 7†				-	99.90*	99.83	99.77	99.77	98.56	97.64
Case 10**					-	*	99.80*	99.80*	98.17*	97.66*
Case 15#						-	99.83	99.83	98.62	97.70
Case 16#							-	99.94	98.68	97.64
Case 17#								-	98.68	97.64
Vaccine A									-	97.82
Vaccine B										-

Strains are referred by the case number.

*Similarities based on a partial *vp60* sequence (981bp long). Vaccines' identification is restrained for privacy.

#Lines highlight samples from the semi-intensive farm.

†Lines correspond to samples originated in the industrial farm.

GenBank accession numbers: Case 0 (KU665601), Case 1 (KU665600), Case 3 (KU665598), Case 7 (KU665597), Case 10 (KU665599), Case 15 (KU665594), Case 16 (KU665596), Case 17 (KU665595).

values (k :3.333, π :0.00340) when compared with the homologous figures obtained independently from the strains of the nv-Farm (k :2.66667, π :0.00153) and the cv-Farm (k :4.667, π :0.00476).

Interestingly, one recombination event (Rm) was detected between nucleotide sites 1272 and 1416, based on 45 pair-wised comparisons of the 7 complete sequences. When analysed independently, no recombination events were detected in any of the 2 sub-populations of sequences.

As expected, at the polypeptide level the variability was lower. All strains originating from the 2 farms exhibited a common polymorphism involving Ile₃₄₇, not shared by the 2 vaccines.

DISCUSSION

RHDV2 RNA was detected in most of the rabbits analysed as 18 (87.5%) of the 21 animals investigated were RHDV2-positive. Vaccine RNA was not identified in any of the tissue samples from which viral RNA was amplified and sequenced. Field strains showed clear nucleotide discrepancies with regard to the sequences of the 2 inactivated vaccines (Carvalho *et al.*, 2017) used. None of the 21 rabbits was positive to classic RHDV, in consonance with the notion that RHDV2 has been replacing the classic RHDV strains in wild and domestic rabbit populations of the Iberian Peninsula (Lopes *et al.*, 2015).

The majority of the dead animals did not show any clinical sign of illness and were in good body condition at the necropsy, which is consistent with an acute disease and sudden death. This finding contrasts with other reports stating that RHDV2 induces a more prolonged disease when compared to RHDV (Le Gall-Reculé *et al.*, 2013; Puggioni *et al.*, 2013), resulting in progressive but extensive liver damage that precedes death. The high level of pathogenicity of the circulating strains is also supported by the elevated mortality rates observed in adults (30-40%) and kits (80%), clearly above the values reported previously in RHDV vaccinated/RHDV2 non-vaccinated farms (up to 20% for adult and 50% for kits (Dalton *et al.*, 2012)).

The macroscopic lesions showed that the lungs and liver were the most affected organs, also in agreement with earlier descriptions (Dalton *et al.*, 2012; Duarte *et al.*, 2015a; Lopes *et al.*, 2015).

Pasteurella multocida, a gram-negative, non-motile *Coccobacillus*, was isolated from the lungs of a RHDV2-negative multiparous doe (case 4). Pasteurellosis, a highly contagious disease transmitted either by direct contact or by aerosols, was not detected in any of the other victims, indicating that this disease was neither related to the serial deaths, nor spread through the rabbitry. The source of this single case was not identified.

No possible cause of death was recognised for the other 2 RHDV2-negative rabbits (cases 14 and 20, Table 1), as none of the tested pathogens was detected.

Given that the disease was detected 1 wk earlier in the nv-Farm, it is likely that the virus spread from there into the neighbouring cv-Farm. The proximity and the sharing of some employees most likely contributed to the dissemination of the virus from the nv-Farm into the cv-Farm. The 100% similarity between the 2 strains obtained in October 2014, when the first cases occurred (case 0 from the nv-Farm, case 1 from the cv-Farm, Table 2), provides molecular evidence of an epidemiological link between the 2 events. This is also corroborated by the lack of diversity between these 2 strains and the strains that circulated in both farms between January and April 2015 (99.69 to 99.90% nucleotide similarity), as well as by the reduced variability observed amongst the strains that circulated later in the farm in 2015 (similarities ranging from 99.43 to 99.94%). The nucleotide variability observed (<0.57%) is below the average value (1.3%) previously described among strains originating from different farms (Le Gall-Reculé *et al.*, 2013). The genetic diversity (π) found among nv-Farm strains (0.00153 ± 0.00044) was significantly lower than in the industrial cv-Farm strains (0.00476 ± 0.00192). This may be related to the higher livestock density in the latter, which favours a higher transmission rate, resulting in a faster accumulation of mutations. The 8 strains did not exhibit any unique or exclusive amino acid variation that could be clearly linked with its putative higher virulence. However, all of them share an Ile at position 347, located in the hyper variable region V2 defined by (Wang *et al.*, 2013). Both vaccines present a different residue at this position. In the large majority of the RHDV2 strains characterised so far, a Thr was mapped at position 347, though a Val or an Ala have also been identified. It is interesting to notice that Ile₃₄₇ is also present in other Portuguese strains previously characterised as originating in the north, centre and south

of mainland Portugal, as well as in the Azores (Duarte *et al.*, 2015a). Most of these strains originated on rabbit farms (KJ683896 and Carvalho *et al.*, unpublished results), but no data was available on the mortality rates induced.

A surprising finding of this study was the death of RHDV2-positive animals that had been vaccinated twice (case 7, Cq 15.27 and 8, Cq 29.08, Table 1), indicating that those animals developed the disease despite vaccination. On the farm, the cold chain for the vaccines is carefully maintained in order to guarantee its preservation. Moreover, all vaccination procedures are carried out systematically by the veterinary assistant according to the protocol, ensuring that all adult animals are vaccinated and that the correct vaccine dose is administered. This greatly reduces the possibility that lack of vaccination or reduced vaccine dose may have been at the origin of the mortality of the vaccinated does. Individual variability to vaccination may have been at the source of those unexpected outcomes. In view of the lower mortality rate induced by RHDV2 when compared to RHDV, detection of viral RNA in apparently healthy animals that recovered from the infection is expected, as genomic RNA or RNA fragments are known to persist for at least 15 wk after experimental infection (Gall *et al.*, 2006, 2007). However, the Cq values of these 2 animals are substantially reduced and too low to represent leftovers from previous infections when compared to the range previously described for RNA and DNA viruses, namely for the blue tongue virus (BTV) (De Leeuw *et al.*, 2015; Barros *et al.*, 2007) and parvoviruses (Duarte *et al.*, 2013). As no animals were sacrificed nor *in vivo* experiments carried out to determine the infectious (viable viral particles) or non-infectious (RNA segments) nature of the RHDV2-RNA, this aspect was not clarified in this study. However, interestingly, they showed no lesions in the liver and lungs and generated a high Cq value (36.14).

The large majority of the RHDV2 RNA-positive rabbits (17/18, 94.4%), including those with Cq values above 30.0 (cases 2, 5, 6), showed typical RHDV2 macroscopic lesions. This fact, along with negative results to other common viral and bacterial pathogens, strongly suggests that all these fatalities, with the exception of case 4, were RHDV2-related.

The death of vaccinated adults indicates that the infection of these animals occurred before an effective immune response could be established, or alternatively, that an ongoing infection hampered the development of an effective immune protection. Exposure to a highly infectious dose, before the vaccine derived protective immune response was fully established, may explain disease development in the adult cases 3 and 7. Despite the virus being able to infect a few vaccinated rabbits from the farm, it did not induce clinical disease in most of the adults that died. Macroscopic lesions were observed in most of the animals but, as the majority of the organs were received frozen, it was neither possible to investigate if typical RHDV2-microscopic lesions were also present in these animals nor to establish a relationship between histo-pathological lesions, viral charge and death for all the animals. This would have been particularly relevant to evaluate the cause of death of the does with lower viral charges.

The significant decrease in the mortality rate observed in the adults after RHDV2 vaccination, which was null after August 2015, compared with the mortality rates observed prior to vaccination (30-40%), exemplified the success of vaccination in controlling the disease.

In the RHDV2 infected kits, a positive association was observed between viral load and the severity of the characteristic anatomo-pathological lesions found in the liver and lungs. Lower Cq values were observed in vaccinated kits (Table 1), suggesting the interference of maternal antibodies with vaccination success, which may have facilitated disease progression (Carvalho *et al.*, 2017).

The time gap between vaccination and casualty varied between 15 d (cases 7, 8, 9, 10 and 11, Table 1) and 121 d (cases 5 and 6, Table 1). Curiously, no trend could be observed between the time gap between vaccination and the Cq value. While no obvious explanation was identified for the lower value of case 3 (Cq 9.53), physiological stress due to recent partum and lactation may have accounted for the progression of the infection in female 7 (Cq 15.27).

The molecular investigation revealed that RHDV2 continued to circulate in the premises for a few months after vaccination. This could be related to the fact that, at any specific time interval, there was always a subset of unvaccinated kits more susceptible to the infection. Infected kits were mostly the offspring of primiparous females, which showed an energy deficit due to simultaneous pregnancy, milk production, and growth, and so were expected to pass lower limited immunity to their litters.

Also interesting was the detection of RHDV2-RNA in an early stage foetus from a RHDV2 positive-doe that had been vaccinated twice, the last shot administered 15 d before death (*results not shown*). A Cq value of 34.08 was obtained from the foetus (*result not shown*), higher than the Cq value (29.08) of the mother, which may suggest the RHDV2 is able to cross the placenta. However, further investigation is required as well as confirmation to rule out foetus contamination with the maternal blood during the uterus opening. To our knowledge, there are no previous reports supporting the congenital infection by RHDV (Xu, 1991).

Neither *Salmonella*, VTEC, *Yersinia*, *Staphylococcus* nor *Listeria* were detected, attesting good sanitary conditions in the rabbitry.

Several risk factors for the introduction of RHDV2 in the rabbitry were identified. The rural surroundings of both farms may have favoured RHDV2 introduction through indirect contact with infected wild species, namely wild rabbits, as no specific protection nets are used. Between 2013 and 2015, a particularly high mortality rate was observed in wild rabbits from legal hunting parks (Dr Fidélia Aboim-Municipal Veterinarian, *personal communication*), the nearest located only 5.3 km away from the farms. According to what has been described in many other regions of the country since 2012 (Abrantes *et al.*, 2013; Lopes *et al.*, 2015; Duarte *et al.*, 2015b), RHDV2 was most probably at the origin of casualties in the wild rabbit population. Therefore, it is possible that RHDV2 transmission occurred from the infected hunting populations into the nv-Farm, via fomites, by human means or arthropods. It has been demonstrated that mosquitoes from the *Culex* genus, active in Portugal from spring to autumn (Alves *et al.*, 2014), play a role in disease dissemination (McColl *et al.*, 2002). Furthermore, the climatic conditions of the area, which has high levels of humidity, create a suitable mosquito habitat.

Likewise, the role of rodents as viral carriers has been supported by the detection of RHDV2 in mammals other than rabbits (Merchán *et al.*, 2011). As the farm is located on agricultural land where rodents are abundant, it is also important to consider their potential role in the transmission of RHDV2 into the rabbitry, although rodent prophylactic measures are carried out.

Another critical point relates to the high resistance of the virus to environmental conditions (Henning *et al.*, 2005). The use of transport vehicles often shared by different rabbit farms may have also accounted for the spreading of the disease locally and across confluent districts.

CONCLUSIONS

The low nucleotide diversity per site ($\pi: 0.00340 \pm 0.00132$) amongst the *vp60* sequences from strains obtained over a 6 mo-period was consistent with a common viral source for the 2 farms. The absolute identity between the strains obtained in both farms during the initial focuses (KU665601 and KU665600) led to recognition of the nv-Farm as the probable source of the virus for the cv-Farm.

Whereas a molecular epidemiological link was established between the 2 farms, no path could be identified as the most probable means for the introduction of the virus in the nv-Farm. However, several risk factors were recognised, relating to the agricultural land where the farms are located, the high mortality of wild rabbits in hunting parks in the same geographic area and the sharing of slaughterhouse vehicles by the rabbitries in the region.

Several months passed before mortality decline was observed following the initiation of vaccination. The time taken before herd immunity could be established undoubtedly highlights the importance of vaccination prior to infection. Several factors may have accounted for the difficulty in reducing and eliminating the virus circulation from the premises, such as the continuous and rapid turnover of the population on intensive farms, which ensures the constant availability of susceptible kits from each new productive cycle, facilitating viral persistence. The putative higher virulence of the strains, supported by the mortality rates observed prior to vaccination, might have required higher antibody titres in the population to impede transmission between animals. No data is known about the immunogenicity of inactivated RHDV2 vaccines when applied as a therapeutic tool to infected populations. However, we believe this dynamic investigation provides preliminary data on the usefulness of vaccination post-infection. While several of these aspects need to be further elucidated, vaccination was proven as an important preventive measure against RHDV2 infection, before rabbitries at risk face this infection.

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