Veterinary Microbiology 198 (2017) 43–50

Contents lists available at ScienceDirect

Veterinary Microbiology

journal homepage: www.elsevier.com/locate/vetmic

Challenges in the rabbit haemorrhagic disease 2 (RHDV2) molecular diagnosis of vaccinated rabbits

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ARTICLE INFO

Article history:
Received 3 April 2016
Received in revised form 29 November 2016
Accepted 3 December 2016

Keywords:
Rabbit haemorrhagic disease virus
RHDV2
RT-qPCR
RHDV2 vaccines
Vaccinated rabbits
Cq values
Molecular diagnosis

ABSTRACT

Molecular methods are fundamental tools for the diagnosis of viral infections. While interpretation of results is straightforward for unvaccinated animals, where positivity represents ongoing or past infections, the presence of vaccine virus in the tissues of recently vaccinated animals may mislead diagnosis.

In this study, we investigated the interference of RHDV2 vaccination in the results of a RT-qPCR for RHDV2 detection, and possible associations between mean Cq values of five animal groups differing in age, vaccination status and origin (domestic/wild).

Viral sequences from vaccinated rabbits that died of RHDV2 infection (n = 14) were compared with the sequences from the commercial vaccines used in those animals. Group Cq means were compared through Independent *t*-test and One-way ANOVA.

We proved that RHDV2 vaccine-RNA is not detected by the RT-qPCR as early as 15 days post-vaccination, an important fact in assisting results interpretation for diagnosis.

Cq values of vaccinated and non-vaccinated infected domestic adults showed a statistically significant difference ($p < 0.05$), demonstrating that vaccination-induced immunity reduces viral loads and delays disease progression. Contrarily, in vaccinated young rabbits higher viral loads were registered compared to non-vaccinated kittens. No significant variation ($p = 0.3824$) was observed between viral loads of non-vaccinated domestic and wild RHDV2-victimised rabbits. Although the reduced number of vaccinated young animals analysed hampered a robust statistical analysis, this occurrence suggests that passively acquired maternal antibodies may inhibit the active immune response to vaccination, delaying protection and favouring disease progression.

Our finding emphasises the importance of adapting kitten RHDV2 vaccination schedules to circumvent this interference phenomenon.

1. Introduction

Six years after its emergence in Europe, rabbit haemorrhagic disease virus 2 (RHDV2) continues to provoke severe economic losses in the industry, to cause great concerns on the conservation of diminished wild rabbit populations and dependent endangered carnivore species, and to affect deeply the cinegetic activity and tourism associated income of some countries.

RHDV2, reported for the first time in 2010 (Le Gall-Reculé et al., 2011a), is classified within the *Lagovirus* genus (Le Gall-Reculé et al., 2011a) along with the close genetically related RHDV, European brown hare syndrome virus (EBHSV) and non-pathogentic lagoviruses (Le Gall-Reculé et al., 2011b). Since its emergence in France (Le Gall-Reculé et al., 2011a), RHDV2 quickly spread throughout neighbouring European countries (Dalton et al., 2012; Abrantes et al., 2013; Le Gall-Reculé et al., 2013; Baily et al., 2014; Westcott et al., 2014)(information on the FLI, 10|21|2013), replacing the previously circulating classical strains (Lopes et al., 2015). RHDV2 was registered outside Europe in Australia (Hall et al., 2015).

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http://dx.doi.org/10.1016/j.vetmic.2016.12.006
0378-1135/

Besides the European rabbit, RHDV2 is also able to infect a few hare species (Puggioni et al., 2013; Camarda et al., 2014). 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 naïve wild population soon after its emergence (Delibes-Mateos et al., 2014).

In view of the alarming impact of RHDV2 in the rabbit industry and in wild rabbit populations, and given the urgency in controlling the disease, RHDV2 inactivated vaccines were developed (Filavac VHD Variant, Filavie Laboratories; Cunipravac variant, Hipra; Novarvilap, Ovejero) and provisionally allowed in the European Union member states under special licenses from the Veterinarian Local Authorities. No data is however available about the immunogenicity and success of the inactivated RHDV2 vaccines when applied as a post-exposure tool to infected populations.

Laboratorial confirmation of RHDV2 is required to assist rabbit farms in disease control. Detection of RHDV2 by molecular methods has undeniable advantages due to their unequalled sensitivity and short execution time, allowing a rapid laboratorial response. A specific RT-qPCR with high sensitivity for RHDV2 detection was recently developed (Duarte et al., 2015a), detecting as few as nine molecules of RHDV2 RNA, and has been in use at INIAV Virology Laboratory since 2014.

The interpretation of RT-qPCR results in non-vaccinated animals undergoing acute disease, particularly when low Cq values are obtained, is usually straightforward. Furthermore, RDH characteristic histopathological lesions are generally present, complementing the molecular diagnosis and allowing the confirmation of RHDV2 as the cause of death. Nonetheless, due to the variable mortality rates described for RHDV2 infections (Le Gall-Reculé et al., 2011a, 2013; Dalton et al., 2012), when low viral loads are obtained differential diagnosis is required since positivity may not necessarily relate to clinical state or fatal outcome.

In addition, given the high sensitivity of nucleic acid amplification-based methods, low amounts of vaccine virus in the tissues of RHDV2 vaccinated animals, may compromise the interpretation of the results and the final diagnosis. Interference of inactivated vaccine RNA on real-time RT-PCR results has been investigated for other viruses to assess the potential associations between recent vaccination and RNA detection in blood or tissues. For blue tongue virus (BTV), it was demonstrated that vaccine viral RNA can reach the blood circulation and the spleen in sufficient amounts to be detected by real-time RT-PCR (De Leeuw et al., 2015). Contrarily, previous studies on the RHDV genome persistence in vaccinated rabbits demonstrated that inactivated vaccine RNA was not detected by RT-qPCR, in samples collected nine weeks after vaccination (Gall and Schirrmeier, 2006). However, in that study no evaluation was undertaken for shorter periods after vaccination. With regards to RHDV field strains, genomic RNA or RNA fragments are known to persist in adult rabbits that overcome experimental infection for at least 15 weeks (Gall et al., 2007). Interestingly, in experimentally infected young rabbits, viral RNA was detected as early as 18 h post inoculation in the liver and spleen, but persisted for a shorter period of only 4 weeks (Shien et al., 2000). Antibodies were developed by these young rabbits between 5 and 7 days post inoculation, with titters correlating well with viremia decreased and viral clearance (Shien et al., 2000), reasserting the important role of immune response in disease control.

In this study, we aimed to clarify if RHDV2 vaccines were detected by the RT-qPCR method developed previously (Duarte et al., 2015a) and if the presence of commercial vaccines in the tissues interfered with the detection of field strains RNA. We also investigated the impact of vaccination on the viral loads during infection, by comparing the Cq values from non-vaccinated and vaccinated infected rabbits. For the vaccinated animals, the algorithm routinely followed to achieve a conclusive RHDV2 diagnosis included the differential diagnosis of pathogenic bacteria, classical RHDV and Myxoma virus, to rule out mixed infections, and the screening by RHDV2-RT-qPCR. Histopathology was performed to confirm the presence of characteristic RHD lesions.

2. Materials and methods

2.1. Samples

Cq (quantification cycle) data from a total of 82 animals that died from RHDV2 infection was analysed in the present study. Vaccinated RHDV2-positive domestic rabbits (n = 14) originated in rabbitries from Portugal mainland, where vaccination had been implemented after the laboratorial confirmation of disease in the premises. These samples were obtained during 2015 for the purpose of this study. For the remaining 68 non-vaccinated rabbits, Cq values were obtained under the same laboratorial conditions, while performing diagnosis between 2014 and 2016. Of these, RHDV2-positive liver samples from non-vaccinated domestic rabbits (n = 29) were received at INIAV directly from the veterinarian assistants of industrial rabbitries or through private laboratories. Wild rabbits (n = 39) were found death in hunting and national parks in Portugal mainland and Azores and sent to INIAV for analysis.

Five groups of animals were defined according to age, vaccination status and domestic/wild origin. Young rabbits corresponded to animals with less than 70 days of age. Group 1 included the domestic vaccinated adult rabbits (n = 11). Group 2 comprised domestic young, born from RHDV2 vaccinated does, which were vaccinated before 35 days of age (n = 3), Group 3 encompassed the domestic non-vaccinated adults (n = 23). Group 4 included domestic young, born from RHDV2 vaccinated does, that had not been vaccinated (n = 6). Group 5 comprised the adult, non-vaccinated wild rabbits (n = 39).

Most RHDV2 vaccinated rabbits (>92%) originated in one rabbitry where a mortality of 30%-40% in adults and 80% in the young was registered in the initial outbreak. For one specimen originated in a second farm, no specific information could be obtained apart from the fact that the animals had been vaccinated after disease onset. After the implementation of vaccination, only a few vaccinated adults and non-vaccinated young died sporadically and mortality decreased to 0% in both age groups.

The time that elapsed between the vaccination of animals from Group 1 and 2 and their casualties, varied between 15 and 121 days.

2.2. Vaccines

The identity of the two RHDV2 commercial vaccines used in the 14 vaccinated rabbits (Groups 1 and 2) is not disclosed for ethical and legal reasons. Instead, these vaccines are hereafter referred to as vaccine 1 and vaccine 2. Among the domestic vaccinated adult rabbits (Group 1), 57.14% of the animals were vaccinated with vaccine 1, 14.29% with vaccine 2 and 28.57% with both vaccines. All the vaccinated domestic young rabbits (Group 2) were vaccinated once with vaccine 2.

2.3. Pathological examination

Necropsies were carried out by the veterinarian assistants at the rabbitries or by the pathologists at the Pathology Laboratory of INIAV.

For histopathological examinations, liver and lung samples were fixed in 10% buffered formalin and embedded in paraffin by

standard procedures. Five micrometer-thick sections were stained with haematoxylin and eosin (H&E) and examined using light microscopy (Cook, 1997).

2.4. Bacteriological examination

Liver and lung samples from the 14 vaccinated animals (Groups 1 and 2) were analysed by standard bacteriological culture, including *Pasteurella* sp., which must be considered in the differential diagnosis of RHD according to the OIE (OIE Technical disease cards). Lung and liver samples macerates were inoculated in MacConkey agar (Oxoid) and Colombia agar (Oxoid) supplemented with 5% of defibrinated sheep blood (Biomerieux) 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).

None of the non-vaccinated rabbits investigated was submitted to bacteriologic examination.

2.5. RNA extraction and virological examination

Liver and lungs samples were homogenized 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. Vaccine RNA was extracted from the aqueous phase of a centrifuged sample (10,000*g* for 10 min) of RHDV2 vaccine 2 and of a classical RHDV vaccine (Cylap, Zoetis), used as a negative control, with the RNeasy blood and tissue kit (Qiagen, Hilden, Germany), according to the recommendations. RNA from RHDV2 vaccine 1 was extracted from 200 μl of a 10× diluted sample (v/v in bidistilled H20), in a BioSprint 96 nucleic acid extractor (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Extracted RNA (10 μl) from tissue samples and the three vaccines were assessed by the RT-qPCR developed by (Duarte et al., 2015a). Screening for RHDV (genogroups G1-G6) 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). The presence of myxoma virus was investigated by qPCR (Duarte et al., 2014), using the FastStart TaqMan Probe Master Kit (Roche, Roche Diagnostics GmbH, Manheim, Germany). For the real time PCR systems described, undetectable Cq or Cq values > 40 were considered negative.

2.6. Nucleotide sequencing analysis and alignments

Amplification of the *vp60* sequences of RHDV2 strains and of the two RHDV2 vaccine strains was accomplished with two pairs of primers, 27F (5′-CCATGCCAGACTTGCGTCCC-3′) and 986R (5′-AACCATCTGGAGCAATTTGGG-3′), 717F (5′-CGCAGATCTCCTCACAACCC-3′) (Duarte et al., 2015b), and RC10R (Tham et al., 1999) generating two overlapping fragments. The One Step (Qiagen, Hilden, Germany) kit was used, following the recommendations of the manufacturer. Sequencing was carried out using a BigDye™ Terminator cycle sequencing kit (Applied Biosystems, Foster City, CA, USA).

The nucleotide sequences of vaccine and field strains were determined on an automated 3130 Genetic Analyzer system (Applied Biosystems, Foster City, CA, USA).

Nucleotide alignments were performed with Clustal omega (http://www.ebi.ac.uk/Tools/msa/clustalo/) (Sievers et al., 2011).

2.7. Statistical analysis

Descriptive statistics and statistical comparison were performed resourcing to the GraphPad Prism, version 5.00 (GraphPad Software, San Diego California USA, www.graphpad.com), for the Cq values and $\log_{10}$ viral loads, obtained for each sample group.

Absolute quantification was calculated from the equation for the linear regression of the method, assuming that the amount of tissue analysed was the same for all samples (≈50 mg) and that the efficiency of the method was close to 100%.

Statistical comparison of mean Cq value and mean $\log_{10}$ viral loads between two specific groups was carried out using the Independent *t*-test, Welch corrected. To verify the difference in the RT-qPCR results between the animal groups, a One-way ANOVA was performed. A p-value < 0.05 was considered to be statistically significant.

3. Results

3.1. Pathology and microbiology of vaccinated RHDV2-infected rabbits

No signs of disease were registered prior death in the vaccinated animals (n = 14, Groups 1 and 2). However, macroscopic lesions suggestive of haemorrhagic disease were observed in all rabbits, including icteric liver and hepatomegaly, hepatic discoloration, lung petechiae and moderate splenomegaly. At the microscopic level, the lesions matched the typical RHD lesions described before (Ohlinger et al., 1993). Necrotic microfoci in liver parenchyma, hepatocyte hyalinization, severe congestion and disseminated intravascular coagulation (DIC) in the small capillaries were registered. All vaccinated rabbits (Groups 1 and 2) tested negative to RHDV, myxoma virus and *Pasteurella multocida,* and positive to RHDV2, from which they died. Bacteriologic examination was not carried out for any of the non-vaccinated rabbits (Groups 3, 4 and 5), where RHDV2 infection was confirmed as cause of death by the low Cq values obtained and the concomitant presence of RHD typical lesions, regardless of the involvement of other pathogens.

3.2. RT-qPCR detects RNA extracted from two RHDV2 vaccines

Serial dilutions of RNA from the three vaccines, obtained as described in subsection 3.4, were tested by the RT-qPCR method (Duarte et al., 2015a). In consecutive dilutions of the two RHDV2 vaccines, a Cq value increase of about three folds was registered *(results not shown)*. RNA from Cylap (Zoetis), a classical RHDV vaccine, was not detected (*results not shown*).

3.3. The strains characterized from the infected-vaccinated rabbits (Group 1 and group 2) differed from the vaccine strains used

The *vp60* nucleotide sequences of the two RHDV2 vaccines were obtained during this study and compared with sequences amplified from RHDV2 vaccinated victimized rabbits (Group 1 and 2), as well as with field strains sequences obtained in our laboratory and available in public databases.

Vaccine sequences are not disclosed here to ensure that any data that the vaccine companies wish to remain private are not made available. Instead, the comparison of the nucleotide sequences of vaccine and field strains is encoded in Fig. 1. The variability between the two vaccines encompassed 38 residues (Fig. 1), of which only three were non-synonymous (residues at positions 9, 347 and 574).

Among the vaccinated animals' strains characterized in this study, residues at positions 405, 450, 912, 1091 (non-synonymous), 1117, 1317, 1491 and 1497 were found conserved differing from the residues found in the two vaccines (Fig. 1, underlined positions). At

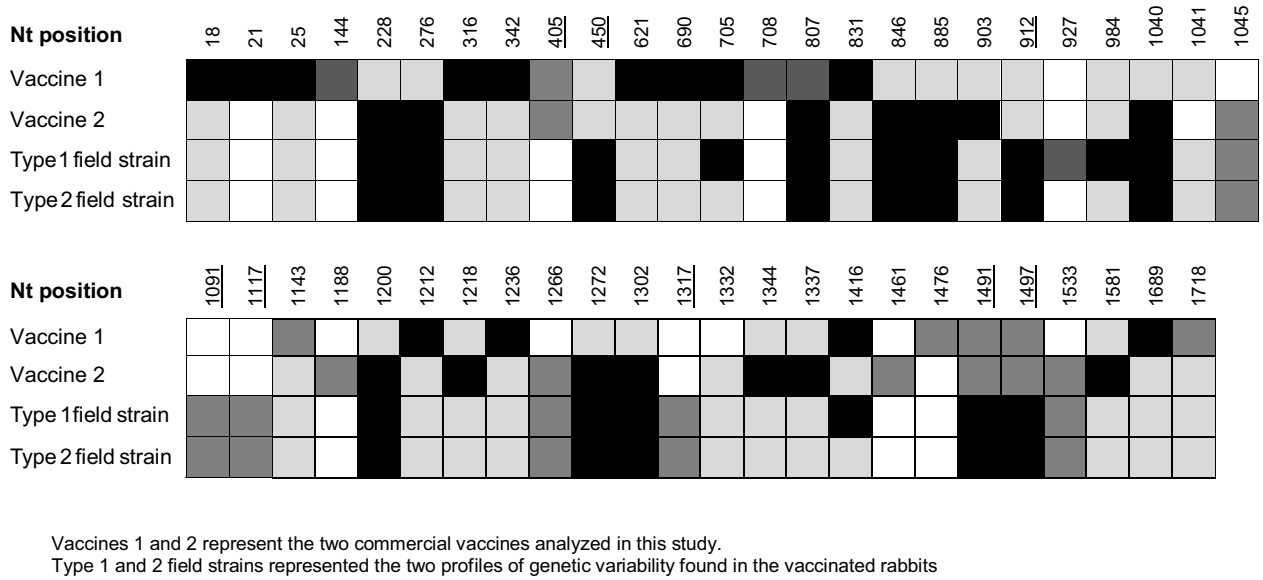

Fig. 1. Schematic representation of the nucleotide variability found between the two vaccine strains used and two consensus field RHDV2 sequences that represent all the strains obtained from vaccinated animals (bottom two). Underlined residues identify the positions that differ between the two vaccines and the field strains from the vaccinated animal group (Group 1 and Group 2). Each nucleotide is represented by a different colour.

the polypeptide level, one vaccine also differed from the field strains in four residues while the other vaccine only diverged in amino acid 364.

When vaccine strains were compared with the sequences obtained from non-vaccinated animals presently available in our laboratory and in the GenBank, one of them showed to be identical to a field strain collected in 2015 in the South of Portugal (*results not shown*) emphasizing the importance of clarifying if vaccine RNA interferes with the RHDV2 molecular diagnosis. Two single nucleotide polymorphisms, located at nucleotide positions 903 (synonymous) and 1041 (non-synonymous), were identified in the other vaccine, allowing its distinction from all field strains presently known.

3.4. Cq variation among the five groups of animals

To assess the impact of vaccination on disease progression and viral loads, the mean Cq values of vaccinated infected animals were compared with those obtained from non-vaccinated rabbits. Cq values are inversely proportional to the amount of target nucleic acid in the sample, meaning that, lower Cqs correspond to higher viral loads (Bustin et al., 2009).

Domestic vaccinated adults showed lower RNA amounts (Group 1, mean Cq 32.01 ± 6.18) than non-vaccinated domestic adults (Group 3) for which a mean Cq value of 15.23 ± 3.82 was obtained (Table 1). This difference was statistically significant ($p < 0.05$, for a 95% confidence interval (CI), Table 2).

A statistically significant difference was also found between the mean Cq values obtained for vaccinated adults (Group 1) and wild rabbits (Group 5) ($p < 0{,}005$, for a 95% CI, Table 2), for which a mean Cq 14.33 ± 3.97 was found, meaning high amounts of RNA were present (Table 1).

The difference found between the mean Cq values, of non-vaccinated domestic adults (Group 3) and wild rabbits (Group 5) was not statistically significant ($p = 0.3824$, for a 95% CI, Table 2).

In regard to young rabbits, the viral loads obtained for the vaccinated young (Group 2, mean Cq of 13.80 ± 2.68) were higher than for the non-vaccinated young rabbits (Group 4, mean Cq of 17.08 ± 4.17) (Table 1). Nevertheless, this difference was also not statistically significant in *t*-test ($p = 0.2026$, for a 95% CI, Table 2) due to the reduced number of samples.

When vaccinated adult (Group 1) and young rabbits (Group 2) were compared, statistically significant differences in mean Cq values were obtained ($p < 0.05$, for a 95% CI, Table 2). Lower viral loads were found in Group 1 (mean Cq of 32.01 ± 6.18) than in Group 2 (mean Cq of 13.80 ± 2.68) (Table 1).

Basic statistics for the $\log_{10}$ viral loads obtained for the five groups, interpolated from the linear regression curve of the

Table 1
Descriptive statistics analysis of the Cq values and $\log_{10}$ viral charges obtained for the vaccinated and non-vaccinated animal groups' considered in this study. The mean and standard deviation were calculated for both indicators.

		Vaccinated		Non-vaccinated		
		Adult domestic (Group 1)	Young domestic (Group 2)	Adult domestic (Group 3)	Young domestic (Group 4)	Adult wild (Group 5)
Cq values	Sample size (n)	11	3	23	6	39
	Mean	32.01	13.80	15.23	17.08	14.33
	Standard deviation	6.18	2.68	3.82	4.17	3.97
$\log_{10}$ viral Loads	Mean	3.41	8.87	8.49	7.88	8.71
	Standard deviation	1.85	0.80	1.08	1.25	1.21

Table 2
Comparative analysis of mean Cq values and viral loads by unpaired *t*-test analysis, Welch corrected. The means of specific groups were compared in order to address the questions' list displayed.

Question addressed	Compared groups		Sig. (α 0.05)[a]	
			Mean Cq	Mean log_{10} viral loads
What is the impact of vaccination in disease progression in adults?	Group 1 Group 3	Vaccinated domestic adults Non-vaccinated domestic adults	$p < 0.0001^*$	$p < 0.0001^*$
Does age of vaccination affects disease progression?	Group 1 Group 2	Vaccinated domestic adults Vaccinated domestic young	$p < 0.0001^*$	$p < 0.0001^*$
What is the impact of vaccination in disease progression in the young?	Group 2 Group 4	Vaccinated domestic young Non-vaccinated domestic young	$p = 0.2026$	$p = 0.2032$
Does age affects the disease progression in non-vaccinated animals?	Group 3 Group 4	Non-vaccinated domestic adults Non-vaccinated domestic young	$p = 0.3110$	$p = 0.3095$
Is disease progression different in domestic vaccinated and wild rabbits?	Group 1 Group 5	Domestic vaccinated adults Non-vaccinated wild adults	$p < 0.0001^*$	$p < 0.0001^*$
Is disease progression different in domestic non-vaccinated and wild rabbits?	Group 3 Group 5	Non-vaccinated domestic adults Non-vaccinated wild adults	$p = 0.3824$	$p = 0.4674$

Groups 3 and 4 passed the Kolmogorov-Smirnov normality test. Group 2 was not assessed due to the sample size.
[*] Statistically significant associations for a 95% CI.
[a] Sig (α 0.05)-statistical significance for a Confidence Interval (CI) of 95%.

RT-qPCR method (Duarte et al., 2015a), were also determined and are shown in Table 1. The mean log10 viral loads are represented in Fig. 2.

When performing One-way ANOVA for the comparison of the five groups, differences in the mean Cq values and mean log_{10} viral loads between groups were statistically significant ($p < 0.05$, Table 3).

A concurrent relation between viral load and severity of the microscopic lesions was observed.

4. Discussion

As expected, the RT-qPCR method (Duarte et al., 2015a) developed to detect RHDV2 field strains, also detected efficiently RNA extracted from the two commercial RHDV2 vaccines. Since the strains used by the vaccine manufacturers' are not publicised, their *vp60* gene sequences were decoded during this study (*results not shown*). The analysis showed that one of the vaccines exhibits a single internal mismatch in the reverse primer, already identified in field strains (Duarte et al., 2015b). Given that the method detects any RHDV2 strain, as long as the target region is conserved, sequencing analysis of the complete *vp60* gene was necessary to differentiate vaccine strains from field strains.

Due to the high sensitivity of the molecular method, inactivated vaccine-derived viral RNA could originate weakly positive RT-qPCR results, if still present in the tissues. Several hypotheses have been considered to explain the detection of inactivated vaccine RNA in animal tissues, namely the unintentional intravasal injection of the

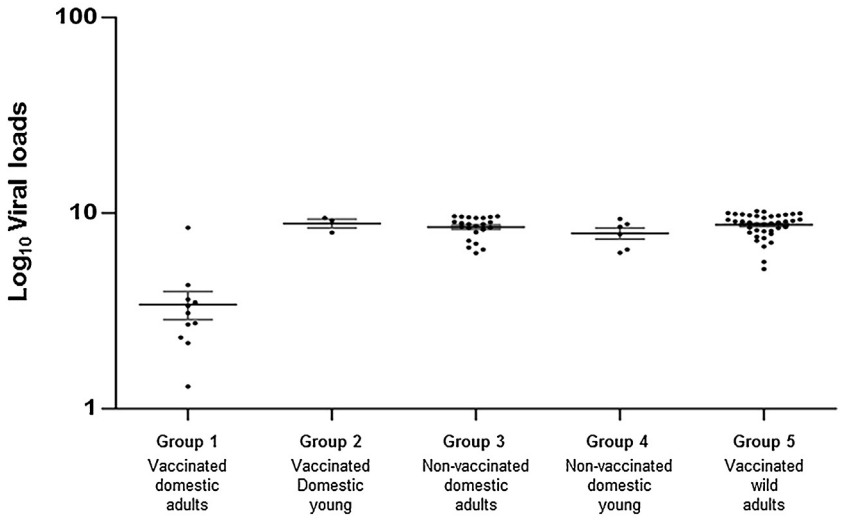

Fig. 2. Log_{10} viral loads obtained for the five groups of animals considered in this study. Dark grey lines and light grey lines show the mean log_{10} viral load and standard deviation calculated for each group.

Table 3
One-way ANOVA comparing the mean Cq values and mean log_{10} viral loads of the five groups of animals specified in this study.

		Sum of squares	df	Mean square	F	Sig (α 0.05)
Cq	Between groups	2897	4	724.30	F (4. 76) = 40.77	$p < 0.0001^*$
	Within groups	1350	76	17.77		
	Total	4248	80			
Log_{10} viral loads	Between groups	258.20	4	64.56	F (4. 77) = 40.04	$p < 0.0001^*$
	Within groups	124.20	77	1.61		
	Total	382.40	81			

df – degrees of freedom; F – F test; Sig-statistical significance for a Confidence Interval (CI) of 95%.
$^*p$-value < 0.05 (statistically significant).

vaccine, the enhanced blood permeability at the site of injection, the systemic distribution of viral RNA via phagocytic cells or the association of inactivated virus with erythrocytes (Eschbaumer et al., 2010; Steinrigl et al., 2010; De Leeuw et al., 2015). For instance, Cq values of above 38.1 obtained from cattle blood samples were associated with BTV inactivated vaccine-derived viral RNA (De Leeuw et al., 2015).

For RHDV2, the amount of inactivated vaccine RNA in the different tissues after vaccination was never investigated. However, regarding the closely related RHDV, Gall and collaborators (2006) observed that, a 100% specific multiplex RT-qPCR assay did not detected inactivated-vaccine derived-RNA, nine weeks after vaccination, probably due to the low titres of the virus in each dose (Gall and Schirrmeier, 2006), whereas RHDV viral RNA could be detected for at least 15 weeks after experimental infection (Gall and Schirrmeier, 2006).

In our study, RHDV2 vaccine RNA was never identified in any of the vaccinated animals not even as earlier as 15 days post-vaccination. In fact, sequencing analysis showed that all the strains characterized from vaccinated infected rabbits clearly differed from the ones from the vaccines (Fig. 1).

Possible associations between mean Cq values (or the corresponding viral loads) obtained from rabbits differing in age, vaccination status and origin (domestic/wild) were explored for significant variability. Results showed that the viral loads in vaccinated adults (Group 1, mean Cq value of 32.01 ± 6.18) were much lower than in both non-vaccinated domestic adults (Group 3, mean Cq of 15.23 ± 3.82) and wild rabbits (Group 5, mean Cq of 14.33 ± 3.97). In these two groups viral loads 100,000× and 200,000× higher, respectively, were calculated. For the vaccinated domestic adults a mean viral load of 4.29E + 02 per mg of liver was obtained, about 80,000× lower than the value previously estimated for RHDV2 infected wild rabbits with the same method (1.5×10^8copies per mg of tissue) (Duarte et al., 2015b). This reduction in the amount of virus in the liver of vaccinated animals may reflect the effect of vaccination on disease progression and clearly proves its usefulness from clinical and epidemiological points of view. Vaccination is considered an effective post-exposure emergency strategy in farms facing RHD outbreaks (OIE Terrestrial Manual, 2016) since immunity develops rapidly, within seven to 10 days after vaccine administration. Protection conferred by vaccination depends on the dose and on the antibody titre developed. Whenever a protective immune response is produced, vaccination prevents infection and/or clinical signs of disease, depending on antibodies titres (Plotkin, 2008). Regarding RHDV, the inhibitory effects caused by the high level of RHDV antibodies in animals that survived experimental RHDV infection were pointed as a possible reason for the failure of experimental transmission of the virus from a highly immunized rabbit to healthy animals (Gall et al., 2007). Those survivors (five among 50) responded with fever and seroconversion showing high antibody titres, and did not developed further RHD specific symptoms or pathological lesions. The rabbits with the highest viral loads in leukocytes (and also in sera) showed the faster normalization of the body temperature, indicating recovery from disease. The viral load decreased during the experiment (Gall et al., 2007). In our study, the 14 infected/vaccinated rabbits originated from a farm where vaccination was performed when the virus was already circulating. The time at which infection took place regarding vaccination is unknown but the time that elapsed from vaccination and death, ranged between 15 and 121 days. Despite this period was quite variable (2 to 12 weeks), the death of the 14 vaccinated adults suggests that an effective immune response could not be established on time. The exposure to a high infectious dose of field strain, when the vaccine-derived protective immune response was not yet fully established, may have accounted for disease development in these adults, which was confirmed by histopathology.

Higher viral charges were obtained both in non-vaccinated domestic adults (Group 3, mean Cq of 15.05 ± 3.5) and in wild adults (Group 5, mean Cq of 16.31 ± 6.69). The range of viral loads in both groups are close to the values previously described (Duarte et al., 2015b) and suggests that disease progression is similar in domestic and wild rabbits. The highest Cq value in the wild rabbits group (Group 5, upper Cq value 33.6) was significantly above the upper value observed in the non-vaccinated domestic adults group (Group 3, upper Cq value 22.58), probably due to the advanced state of putrefaction of some specimens (n = 4). When these poor quality samples were excluded, the mean Cq value for wild adult rabbits dropped to 14.33 ± 4.0, approaching the homologous value of the non-vaccinated domestic group (mean Cq 15.05 ± 3.5).

Regarding the young rabbits (Groups 2 and 4), a relation between vaccination and reduction of viral amounts was not observed. On the contrary, the viral loads found in vaccinated young rabbits were higher (Group 2, mean Cq 13.80 ± 2.68) than in non-vaccinated kittens (Group 4, mean Cq of 17.08 ± 4.17). In view of the higher mean Cq values observed in non-vaccinated young it is tempting to speculate that a higher antibody response was elicited in these animals where maternal antibodies were not subtracted by vaccine antigens, which suggests that interference with maternal antibodies may impair vaccination success, facilitating disease progression.

Humoral immunity is critical to protect rabbits from RHD (Argüello Villares, 1991; Laurent et al., 1994) and maternal IgG antibodies, acquired during late pregnancy through the placenta and, later on, via colostrum (Lorenzo Fraile, *personal communication*), may be relevant for young rabbits' resistance to RHDV (Cooke, 2002). Rabbit kittens IgGs' can persist for up to 12 weeks after birth (Lengahus C, *unpublished,* cited by (Cooke, 2002)), showing a progressive decline as age and body weight increase (Cooke, 2002). However, maternal antibodies' impact on the RHDV2 vaccination efficacy was never evaluated, but should be taken into account to assure immunization success in RHDV2 vaccination programmes.

Non-vaccinated young, born from RHDV2 vaccinated mothers, showed lower viral loads (Group 4, mean Cq of 17.08 ± 4.17) than non-vaccinated adults (Group 3, mean Cq of 15.05 ± 3.5), in accordance with previous studies (Strive et al., 2010). Although the number of animals available from this group was extremely reduced due to the infrequent fatal outcome in vaccinated animals, this difference may reflect the partial protection conferred by RHDV and RHDV2 immunized mothers. In the RHDV2 infected kittens, a positive association was observed between viral load and the severity of the characteristic anatomopathological lesions found in liver and lungs.

5. Conclusions

One important finding of this study was that, although the RHDV2 RT-qPCR is able to detect vaccine RNA extracted directly from the vaccine suspensions, in animals that had been vaccinated as recently as 15 days before, vaccine RNA did not interfere with the detection of field strains. This piece of information is extremely useful for the overall interpretation of laboratorial results in vaccinated animals, and is especially important as the nucleotide sequences of the vaccines used are usually unknown.

Information on the viral loads is an important addition to qualitative diagnostics. Analysing Cq values obtained in different groups defined according to age, vaccination status, and domestic/wild, allowed us to observe a concurrent relation between Cq values and vaccination in the domestic adults group. Also, no differences in the severity of the disease in domestic and wild rabbits were reported and, accordingly, no significant difference was observed between the viral loads of non-vaccinated domestic and wild rabbits ($p = 0.4674$).

Although the difference was not statistically significant due to sample size, in agreement with (Duarte et al., 2015b), and as it was also described for RHDV (Strive et al., 2010), the present analysis suggest that higher mean viral charges are usually obtained in the non-vaccinated domestic adults than in non-vaccinated young rabbits. Previous studies suggest that proper immune response induced by vaccination may reduce viral titres and the amount of RNA detected by molecular means (Gall and Schirrmeier, 2006). Diagnose based on the detection of low levels of RHDV or RHDV2- RNA should therefore be complemented by histopathology to elucidate infection status. Differential diagnosis with other relevant pathogens should also be considered.

We believe this preliminary investigation provides for the first time, laboratorial data on the effect of post-infection vaccination on molecular diagnosis outcome. The reduced number of samples available from rabbits that died after vaccination (n = 14), constituted the major limitation of this investigation. Although not statistically significant, the trends suggested by the data sets of vaccinated and non-vaccinated young, are in accordance with the notion that early vaccination against RHDV2, similarly to many other viruses such as canine parvovirus (Waner et al., 1996), may be counter-productive due to the presence of the maternal antibodies in the offspring. Vaccination programmes should take into account the inhibitory effect of these antibodies on active immunization that may compromise the success of vaccination of young animals.

Further investigations will have to be conducted on the decay of maternal antibodies and the extent to which they interfere with the active humoral response induced by RHDV2 vaccination in the young, in order to establish more efficient vaccination programmes for the different age groups.

Acknowledgments

The authors thank to Dr. José Monteiro (Coren, SA) and Dr. Carla Afonso and Dr. João Pacheco (Segalab, Laboratório de sanidade animal e segurança alimentar, SA) for providing us most of the samples of vaccinated animals. Our acknowledgements go also to Dr Maria do Carmo Palma Caetano (Serviços de Alimentação e Veterinária da Região do Alentejo), Dr Lucília de Azevedo Mendes (Divisão de Gestão e Autorização de Medicamentos Veterinários, Direção-Geral de Alimentação e Veterinária, Ministério da Agricultura e do Mar), for the commercial vaccines. The authors are also thankful to Maria João Teixeira, Fátima Cordeiro and Ricardino Ferreira for their technical assistance. This study was partially funded by the Fundação para a Ciência e Tecnologia (FCT), grants SFRH/79225/2011 and UID/AGR/00115/2013.

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