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Authors: Carlos Calvete, Manuel Mendoza, Ana Alcaraz, María P. Sarto, María P. Jiménez de Bagüés, Antonio J. Calvo, Fernando Monroy, Jorge H. Calvo

PII: S0378-1135(18)30109-3
DOI: https://doi.org/10.1016/j.vetmic.2018.04.018
Reference: VETMIC 7943

To appear in: *VETMIC*

Received date: 24-1-2018
Revised date: 11-4-2018
Accepted date: 11-4-2018

Please cite this article as: Calvete C, Mendoza M, Alcaraz A, Sarto MP, de Bagüés MPJ, Calvo AJ, Monroy F, Calvo JH, Rabbit haemorrhagic disease: cross-protection and comparative pathogenicity of GI.2/RHDV2/b and GI.1b/RHDV lagoviruses in a challenge trial, *Veterinary Microbiology* (2010), https://doi.org/10.1016/j.vetmic.2018.04.018

Rabbit haemorrhagic disease: cross-protection and comparative pathogenicity of GI.2/RHDV2/b and GI.1b/RHDV lagoviruses in a challenge trial.

Carlos Calvete [a,*], Manuel Mendoza [a], Ana Alcaraz [b], María P. Sarto [a], María P. Jiménez de Bagüés [a], Antonio J. Calvo [c], Fernando Monroy [c], Jorge H. Calvo [a,d].

[a] Animal Production and Health Department, Agrifood Research and Technology Centre of Aragon (CITA). Agrifood Institute of Aragon–IA2 (CITA-Zaragoza University), Avda. Montañana 930. 50059 Zaragoza, Spain.

[b] College of Veterinary Medicine, Western University of Health Sciences, Pomona, California, 91711 United States of America.

[c] Animal Health Management, TRAGSATEC, C/ Julián Camarillo 6 A, 28037 Madrid, Spain.

[d] ARAID, 50004 Zaragoza, Spain.

* Corresponding author. Tel.: +34 976716453; fax: +34 976716435. E-mail address: ccalvete@aragon.es (C. Calvete).

Highlights

- Both GI.2 and GI.1b lagoviruses caused acute forms of rabbit haemorrhagic disease
- Cross-protection was partial and not identical between the two viruses
- Case fatality from GI.2 varied between individuals due to as yet unknown parameters
- Case fatality from GI.1b was related to age with a pattern never described before

Abstract

European rabbits (*Oryctolagus cuniculus*) are severely affected by rabbit haemorrhagic disease (RHD). Caused by a lagovirus, the disease leads to losses in the rabbit industry and has implications for wildlife conservation. Past RHD outbreaks have been caused by GI.1/RHDV genotype viruses. A new virus belonging to the GI.2/RHDV2/b genotype emerged in 2010, quickly spreading and replacing the former in several countries; however, limited data are available on its pathogenicity and epidemiological factors. The present work extends these issues and evaluates cross-protection between both genotypes. Ninety-four and 88 domestic rabbits were challenged with GI.2/RHDV2/b and GI.1b/RHDV variant isolates, respectively. Cross-protection was determined by a second challenge on survivors with the corresponding strain. Mortality by GI.2/RHDV2/b was highly variable due to unknown individual factors, whereas mortality by GI.1b/RHDV was associated with age. Mortality in rabbits < 4 weeks old was 84%, higher than previously reported. Cross-protection was not identical between the two viruses because the ratio of mortality rate ratios for the first and second challenges was 3.80±2.68 times higher for GI.2/RHDV2/b than it was for GI.1b/RHDV. Rabbit susceptibility to GI.2/RHDV2/b varied greatly and appeared to be modulated by the innate functionality of the immune response and/or its prompt activation by other pathogens. GI.1b/RHDV pathogenicity appeared to be associated with undetermined age-related factors. These results suggest that GI.2/RHDV2/b may interact with other pathogens at the population level but does not satisfactorily explain the GI.1b/RHDV virus's quick replacement.

Keywords: Lagovirus; *Oryctolagus cuniculus*; Rabbit haemorrhagic disease; RHD; RHDV; RHDV2/b

1. Introduction

Rabbit haemorrhagic disease (RHD) is caused by caliciviruses of the species *Lagovirus europaeus* (GI genogroup), which mainly affect European rabbits (*Oryctolagus cuniculus*). Rabbit haemorrhagic disease is rapidly fatal, highly infectious and characterized by acute necrotising hepatitis. It provokes high mortality rates (> 90%) in adult rabbits, but young kits are generally naturally resistant to lethal infection. First described in China in 1984, RHD viruses have spread worldwide, and at present, the disease is considered endemic in many European countries, Australia and New Zealand, being a constant threat to domestic and wild rabbits (reviewed in Abrantes et al., 2012).

Until recently, and following the new nomenclature proposed by Le Pendu et al. (2017), all RHD-viruses belonged to the GI.1 genotype. In 2010, however, a new virus causing RHD outbreaks in France was identified (Le Gall-Reculé et al., 2011). This new lagovirus, referred to as the RHDVb variant (Dalton et al., 2012) or RHDV2 (Le Gall-Reculé et al., 2013), differs phylogenetically from other lagoviruses (Le Gall-Reculé et al., 2013), being included in the new genotype, GI.2 (Le Pendu et al., 2017). This new virus, GI.2/RHDV2/b (hereafter GI.2), causes lower average mortality than GI.1-genotype viruses, although induced mortality in very young rabbits is higher than that caused by GI.1.

GI.2 has since rapidly spread throughout other European countries, replacing the previously circulating viruses, particularly those belonging to the GI.1b/RHDV variant (hereafter GI.1b) (Le Gall-Reculé et al., 2013; Dalton et al., 2014; Calvete et al., 2014; Mahar et al., 2018), and is also present in Australia (Hall et al., 2015). This rapid widespread virus has again created a new threat to rabbit farming and a negative ecological impact on wild rabbit populations.

Despite GI.2's current widespread distribution, limited data are available on its pathogenicity (Le Gall-Reculé et al., 2013) and the factors involved in it rapidly replacing the previously circulating GI.1b virus. To extend the current knowledge on these issues, the present work determined the cross-protection between GI.2 and GI.1b, comparing their pathogenicity across rabbit age. The main goal was to elucidate whether pathogenic and/or antigenic differences between both viruses could involve any selective advantage of GI.2 over GI.1b in the field.

2. Material and methods

2.1. Experimental infections

Minimal disease level New Zealand White laboratory rabbits were provided by Granja San Bernardo (Tulebras, Spain). Neither the rabbits nor their parents were myxomatosis- or RHD-vaccinated.

Given the lack of previous data, sample size calculation for comparing two proportions (based on Z-testing) was used to approximate the sample size needed to detect differences between mortality rates caused by the same virus at the first and second challenges (the second was performed on rabbits that survived the first challenge by the other virus). A rounded 40% mortality rate by GI.2 from the first challenge was assumed from data reported by Le Gall-Reculé et al. (2013) in selected commercial New Zealand domestic rabbits. A 90% mortality rate was assumed for GI.1b at the first challenge for rabbits older than 8 weeks. Sample size calculations were conservative to detect halving in mortality rates from the first to second challenges at 80% statistical power and a 0.1 two-tailed significance level.

After estimating minimum sample sizes, rabbits were separated into experimental age batches based on provider availability and RHD susceptibility, particularly to the GI.1b

virus. To reduce within-batch correlations, members from the same litter or breeding cage were not allowed in the same experimental age batch.

In total, 94 rabbits distributed in 8 age batches and 65 rabbits distributed in 6 age batches were first challenged with GI.2 and GI.1b viruses, respectively (Table 1). To ensure that enough rabbits survived to be challenged with GI.2, given the high mortality by GI.1b in rabbits older than 8 weeks, most of the animals that were first challenged with this virus (60 of 65) were younger. To reduce the number of rabbits used for ethical reasons, data from 4 batches of adult rabbits (13-14.7 weeks of age) that were first challenged with the same GI.1b isolate in preliminary assays with identical protocol (not previously published) were added to the dataset to represent adult rabbit mortality.

Two virus inocula were used for challenges. GI.1b inoculum was prepared from an isolate obtained from a naturally RHD-dead wild rabbit (*Oryctolagus cuniculus*) collected in the Huerva River Valley in 2008 in Zaragoza province (Spain) (GenBank acc. numbers KY498582 and MH159721 for the *VP60* capsid protein and the *RdRp* gene respectively). The virus was passaged twice in laboratory rabbits, and a 20% liver homogenate in PBS was prepared from the liver of a laboratory rabbit dead from acute RHD. The tissue suspension was centrifuged, and the supernatant was diluted 100-fold in PBS, yielding an oral viral suspension of $5x10^{3.5}$ LD50/ml for adult rabbits which was used for experimental challenges. Viral suspension was titrated by the Reed and Muench method (Reed and Muench, 1938), infecting 5 rabbits per serial dilution (10^{-1} to 10^{-6}) of the supernatant (data not published). GI.2 inoculum (GenBank acc. numbers MG022138 and MH159722 for the *VP60* capsid protein and the *RdRp* gene respectively) was prepared following the same procedure from a naturally RHD-dead wild rabbit collected in 2013 from an experimental enclosed wild rabbit population in

Zaragoza province. The first GI.2 viral detection in this experimental population occurred in 2011, which quickly replaced the previously circulating GI.1b virus (Calvete et al., 2014). Given the substantial genetic diversification reported during last years for lagoviruses, phylogenetic analyses of VP60 and RdRp gene sequences (Dalton et al., 2018) were used to confirm that both isolates were not recombinants, at least in the subgenomic promoter region most prone to recombination events (Lopes et al., 2015). (Appendix A: Supplementary material).

The GI.2 viral suspension was not titred; however, because livers from rabbits that died from RHD by either GI.2 or GI.1b contain similar viral amounts (Le Gall-Reculé et al., 2013) and an identical protocol was used to prepare both viral suspensions, it is assumed that a similar viral dose was administered in both inoculums.

Experimental infections were performed under negative pressure in BSL2 (CITA) experimental facilities with filtered air. Rabbits were reared in separated individual cages with free access to water and food (rabbit pellets provided by Granja San Bernardo). To avoid cross-contamination, each experimental batch was placed in a different negatively pressurized room. Before the first challenge (day 0), a blood sample from each rabbit was taken from the marginal ear vein to confirm seronegativity against RHD with a commercial indirect ELISA test primarily developed to detect anti-GI.1b Ig G antibodies (Ingezim Rabbit. INGENASA Lab., Madrid, Spain). Each rabbit then received 0.2 ml of the appropriate viral suspension (GI.2 or GI.1b) orally.

After the first challenge, rabbits were monitored for clinical changes and mortality at least 4 times daily for 35 days post-infection. During this period, a blood serum sample was obtained from each rabbit on the 7^{th}, 21^{st} and 35^{th} days post-infection to monitor seroconversions using the same commercial indirect ELISA test. Additional samples of

EDTA blood were obtained from each rabbit in the 3.4, 4.5 and 10.5-week-old batches infected with either GI.1b or GI.2 (and the 15.5-week-old batch infected with GI.2) on the 0, 7th, 21st and 35th days post-infection to monitor variations in the following haematological parameters: haematocrit value (HCT), red blood cell counts (RBC), haemoglobin concentration (HGB), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC) and differentiated neutrophil, eosinophil, basophil, lymphocyte, monocyte and platelet counts. Analyses were performed by Albéitar Veterinary Laboratory (Zaragoza, Spain) by flow cytometry (cytometer Sysmex XT-2000iV). Liver transaminase, alanine aminotransferase (ALT) and aspartate aminotransferase (AST) concentrations were also evaluated in the serum for the same days and age batches using an autoanalyzer GERNON (RAL®) following reference procedures from the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC).

To estimate cross-protection between both viruses on the 35th day post-infection, a second challenge was conducted on rabbits that survived the first, following identical protocol. Thus, rabbits initially surviving GI.1b were then infected with GI.2 and vice versa, except 8 rabbits that were euthanized on the 35th day post-infection for logistic reasons (Table 1). After the 2nd infection, rabbits were monitored for 35 days for clinical changes and mortality. During this period, a blood serum sample was obtained from each rabbit on the 7th, 21st and 35th days post-infection to draw seroconversion using the same commercial indirect ELISA test. Haematological parameters and transaminase concentrations were not monitored after the second challenge.

Dead rabbits, as well as those that survived to the end of the experiment and were then humanely euthanized, were necropsied and examined for macroscopic lesions. Then, the spleen and liver weight ratios to body weight were estimated. Liver samples were

collected to detect RHD virus by duplex real-time PCR (duplex qPCR). In addition, tissue samples from the liver, spleen, kidneys, lung and small intestine were collected from 42 rabbits to compare histopathological findings among those that had died by GI.2 (n = 13) at first or second challenge, those dead by GI.1b (n = 16) at first or second challenge (including 3 animals < 4-weeks-old) and surviving rabbits (n = 13). Tissue samples were fixed in 10% buffered formalin and routinely processed and stained with haematoxylin-eosin for histological examination.

In addition to experimentally infected animals, 11 4.5-week-old rabbits were used as controls. Three were euthanized after reception and used as controls for histopathological comparisons with young rabbits. The remaining 8 were maintained in conditions identical to the infected rabbits for 70 days. During this period, a serum sample from each rabbit was obtained on the 0, 7^{th}, 21^{st}, 35^{th}, 42^{nd}, 56^{th} and 70^{th} days as seroconversion and transaminase concentration negative controls. EDTA blood samples were obtained on the same days, simultaneously with the experimentally infected rabbits to control natural variations in haematological parameters across the survey. Finally, the control rabbits were euthanized, and their samples were used as controls for histopathological comparisons with the older rabbits.

All animal experiments were performed in compliance with the provisions of Spanish national and European laws (Law 32/2007, modified 6/2013, and RD 53/2013) and approved by the CITA Ethical Committee for Animal Experimentation (protocol 2014-18).

2.2. Duplex real-time PCR (duplex qPCR)

Given the recent emergence of GI.2 and that the goal of the background research projects was to study RHD epidemiology before and after the spread of GI.2, a duplex

qPCR procedure was developed to detect both GI.1b and the new GI.2 genotype virus in a single analysis.

Total RNA was extracted from 25 mg of liver tissue using TRI REAGENT (Sigma LifeScience, Madrid, Spain) according to the manufacturer's instructions. For duplex qPCR, first-strand cDNA was synthesized from 1 µg of RNA using the SuperScript III reverse transcriptase kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions with the specific primer, 5'-CCAATTGTTACTGGCAGTGGT-3', described by Moss et al. (2002). This primer is located at positions 1470–1490 in the *VP60* gene.

Primers and probes were designed based on conserved regions after aligning a 524-bp fragment of the *VP60* gene (corresponding to nucleotides 871 - 1394 in the VP60 gene according to the GenBank acc. Numbers, KY498582 and KY498543, for GI.1b and GI.2, respectively) from GI.1b and GI.2 isolates taken from Spain, France, Portugal and Italy. Specific molecular beacon probes were used for each virus. Primers and molecular beacon probes were designed by Mycrosynth (Mycrosynth, Balgach, Switzerland).

Duplex qPCR was performed using an ABI Prism 7500 platform (Applied Biosystem, Madrid, Spain). Amplification was performed in 10-µl volume reactions containing 5 µl of Premix Ex Taq (2x) master mix (Takara, Japan), 0.1 µl of Rox ref dye II, 0.2 and 0.4 µM of each primer and probe, respectively, 3.1 µl of RNase- and DNase-free water (Applied Biosystems, Madrid, Spain), and 1 µl of cDNA (500 ng). Thermal cycling conditions included one cycle at 95°C for 30 s to activate Taq polymerase and 50 cycles of cDNA amplification (95°C for 5 s and 59°C for 34 s). All samples were amplified in duplicate in the same run. More extensive information on development, specificity and

efficiency of the duplex qPCR is available in the supplementary material with this article (Appendix A: Supplementary material).

2.3. Statistical analyses

2.3.1. Lesions and blood parameters

Variations in spleen and liver weight ratios to body weight were explored by fitting linear regression models in which rabbit age at death (in weeks), group and time to death (in hours) were included as predictor variables in the initial model. Age was included due to its physiological linear inverse relationship with both ratios. For group variables, data were divided into five groups: rabbits that died by GI.2 or GI.1b at the first or second challenge and the control and euthanized-survivor rabbits. Control and euthanized rabbit data were mixed since they did not differ in preliminary analyses. For analytical purposes, time to death of this group was assumed to be zero. Backward stepwise selection was applied to obtain the final model. Variables were retained in the model if the *F* statistic p value was ≤ 0.1.

Changes in blood parameters (haematological parameters and liver transaminases) of the rabbits that survived the first challenge were evaluated adjusting a repeated measures ANOVA model to data recorded at the 0, 7^{th}, 21^{st} and 35^{th} days post-infection for each blood parameter. As predictors, a variable with the following three levels: infection with GI.2, infection with GI.1b and no infection (data from non-infected control rabbits) was included in addition to its interaction term with the within-effect factor (time). Because physiological variation in these parameters is associated with age, the 3.4- to 4.5-week-old and 10.5- to 15.5-week-old rabbits were analysed separately. Non-infected control rabbit data from days 0 to 35 and days 35 to 70 were used as the control values, respectively.

2.3.2. Mortality and cross-protection

Cross-protection was evaluated by fitting a Poisson regression model to mortality data with log-link function, in which the virus, the challenge and their interaction were included as binomial predictor variables. To reduce the confounding effect of rabbit age, mortality data for rabbits < 8 weeks old infected with GI.1b at the first challenge were not included.

Putative factors associated with mortality were explored by fitting a Poisson regression model, with log-link function, to mortality data from the rabbit subset sampled for haematological parameters. Sex, age batch (both as categorical variables) and haematological parameters measured at day 0 (or the 35th day for the second challenge) were included as predictor variables in the initial model. Backward stepwise selection was used, and variables were retained in the final model if the Wald statistic p value was ≤ 0.1. A separate Poisson model was fitted to each virus and challenge. For the second challenges, absorbance values of the indirect ELISA test on the 35th day after the first challenge were also included in the predictor variables set as a surrogate of antibody concentration against RHD viruses.

2.3.3. Time to death

Student's *t*-test was used to compare mean time to death (in hours) between rabbits infected with GI.2 or GI.1b at the first challenge and to test differences between rabbits infected with the same virus at the first or second challenge. Homogeneity of variances was assessed to apply the correct *t*-test.

Factors related to time to death were explored by fitting a linear regression model to time to death values recorded from the rabbit subset sampled for haematological parameters. Sex, age-batch (as categorical variables) and haematological parameters

measured at day 0 (or the 35th day for the second challenges) were included as predictor variables in the initial model. Backward stepwise selection was used, and variables were retained if the *F* statistic p value was ≤ 0.1. A separate regression model was fitted to each virus and challenge. For the second challenge, ELISA test absorbance values on the 35th day were also included as predictor variables.

2.3.4. Duplex qPCR

Non-parametric Spearman's correlation, a two-tailed difference between two proportions test and two-tailed Fisher´s exact probability test were used to explore associations within duplex qPCR results of surviving rabbits.

3. Results

3.1. Histopathology, seroconversion and blood parameters

In all, 253 experimental infections, 182 at the first and 71 at the second challenges, were performed. All rabbits that died did so from acute forms of RHD, except for 2 10.5-week-old rabbits that survived to the first challenge with GI.1b. These two animals developed a chronic form of the disease, with severe jaundice, anorexia and concomitant bacterial infections and were euthanized 10 days after the challenge.

At necropsy, all RHD-dead rabbits showed typical and similar gross lesions, with minimal or moderate generalized congestion. Tracheal mucosal reddening, pulmonary multifocal haemorrhages and severe congestion were noted. Livers were brittle with a generalized marked lobular pattern, while the spleens were dark red, with occasional splenomegaly.

As expected, the major histopathological lesions present in all 29 rabbits that died by RHD were acute hepatitis with minimal to moderate multifocal necrosis, inflammation,

hepatic cord dissociation, bile duct proliferation and lobular atrophy. Hyperaemia and thrombi were also present in the lungs, kidneys or spleen. Most animals showed spleen follicle depletion, haemorrhaging, tubulonephrosis and tubular necrosis in the kidneys. Less frequent lesions (n = 7) were found in the small intestines, with individual cell necrosis and Peyer's patch depletion. Lesion occurrence was not associated with rabbit age, time of death, viral genotype or challenge (data not shown). Rabbits that survived both challenges show no clinical signs, and no gross or histopathological lesions were found.

The final regression model fitted to the spleen weight to body weight ratio (R^2 = 73.50%; n = 112; F = 151.17; $p < 0.001$) showed that the spleen ratio was directly related to time to death (parameter ± SE: 0.001 ± 0.0001; t = 10.84; $p < 0.001$) but not with the virus. This relationship, corrected by the age effect, is shown in Fig. 1 and shows a similar distribution trend over time for rabbits dead from both GI.2 and GI.1b. No change in liver weight was associated with RHD.

Positive seroconversion was detected in all rabbits after the first challenge, demonstrating that all surviving rabbits were successfully infected. High ELISA absorbances were recorded for all rabbits until the end of the assay (Fig. 2). Absorbance values were consistently lower for GI.2, probably due to the lower sensitivity of the commercial ELISA test for this genotype, as the test was initially developed to detect anti-GI.1b antibodies.

Repeated measures ANOVA models fitted to blood parameter variations showed no significant differences ($p > 0.5$ in all cases) in changes along the assay among non-infected control rabbits and survivors 3.4-4.5 weeks old first challenged with GI.2 or

GI.1b (*n* = 16 and 9, respectively) or those 10.5-15.5 weeks old infected with GI.2 (n = 8).

3.2. Mortality and cross-protection

The overall mortality rates at first challenge were 0.43 (40/94) and 0.67 (59/88) among rabbits infected with GI.2 and GI.1b, respectively. By experimental batches (Fig. 3), mortality from GI.2 at the first challenge was highly variable, ranging from 0.75 (6/8) to 0 (0/15), with no specific age-related pattern. Conversely, mortality by GI.1b exhibited a marked, but unexpected, age pattern. Typical for this viral genotype, mortality was as high as 0.93 (26/28) in rabbits ≥ 8 weeks old and as low as 0.34 (12/35) in rabbits between 4 and 8 weeks old. However, the mortality rate in younger rabbits (< 4 weeks) was 0.84 (21/25), notably higher than expected.

Regarding second challenges, all rabbits were infected at ages over 8 weeks. Two animals (9.5 and 11 weeks old) were euthanized just after the second challenge with GI.1b because of accidental spinal cord injuries, and were excluded from the assay. Total mortality rates were 0.07 (2/27) and 0.61 (27/44) in rabbits infected with GI.2 and GI.1b, respectively. Therefore, mortality caused by GI.2 was 5.74 (CI 95%: 1.48-22.25) times higher at the first than the second challenge, in contrast to mortality caused by GI.1b, which had a mortality rate ratio of 1.51 (CI 95%: 1.17-1.95) for rabbits ≥ 8 weeks old.

The Poisson regression model (Pseudo-R^2 = 7.91%; n = 193; Wald χ^2 =52.35; $p < 0.001$) confirmed that the mortality caused by GI.1b was 2.18±0.29 (±SE) times higher than that by GI.2 ($z = 5.95$; $p < 0.001$) and that the mortality at the second challenge was 0.17±0.12 times the mortality at the first ($z = -2.52$; $p = 0.012$). Interestingly, the interaction term showed that the mortality rate between the first and second challenges

was 3.80±2.68 times higher for GI.2 than for GI.1b (z = 1.89; p = 0.058), thus confirming that the cross-protection was asymmetric, i.e. it was not identical between both viruses.

Regarding mortality-associated factors, the Poisson model obtained for rabbits first challenged with GI.2 (Table 2), showed that it was directly correlated with MCH and lymphocyte count but inversely correlated with MCHC and monocyte count. However, some degree of collinearity between experimental batches and haematological parameters was detected (Table 3), especially because the 10.5-week-old rabbit batch had the highest mean monocyte count and the lowest mortality rate (0%). To evaluate the effect of collinearity on these results, the same model was re-fitted, adding the batch as a predictor variable. The new model fit the differences in mortality among batches, showing statistically significant differences between the 10.5-week-old rabbit batch and the remaining batches ($p < 0.001$ in all comparisons). However, this model also estimated similar and statistically significant mortality rate ratios for the four haematological parameters as in the first model (Table 2), confirming that the haematological parameter relationships with mortality were not confounded by batch collinearity.

Conversely, the Poisson model fitted to mortality by GI.1b at the first challenge only retained the batch as a predictor variable (Pseudo-R^2 = 8.38%; n = 34; Wald χ^2 = 8.61; p = 0.013), mainly due to differences between mortality rates in the 3.4-week-old (highest mortality) and 4.5-week-old (lowest mortality) batches (mortality rates ratio ± SE: 3.27 ± 1.42; z = 2.72; p = 0.006). No collinearity between haematological parameters and batches was observed as a putative confounding factor.

For the second challenges, the model fitted to mortality by GI.2 only retained the monocyte count (Table 2), again indicating that the monocyte count was inversely correlated with mortality. For GI.1b, no predictor variable was retained in the final model.

3.3. Time to death

Time to death among rabbits first challenged with GI.2 showed no evidence that supported any pattern associated with rabbit age (Fig. 4), showing a mean time to death of 52.77 hours after infection (SD = 11.72; range 35.50-83.00). Data for rabbits that were first challenged with GI.1b, however, showed a clear age-related pattern, with a higher mean time to death in the youngest batches. Rabbits under 5 weeks old showed a mean time to death of 116.72 hours (SD = 14.71; range 78.00-145.00) compared to a mean time to death of 50.56 hours for older rabbits (SD = 27.89; range 33.00-177.00). The last value was similar to that of rabbits first challenged with GI.2 (Student´s *t*-test = 0.21; df = 35.96; p = 0.829).

At the second GI.2 challenge, only two rabbits died at 74.50 and 99.80 hours after infection (mean = 87.12; SD=17.85), whereas rabbits killed by GI.1b exhibited a mean time to death of 65.93 hours (SD = 39.10; range 31.00 – 229.50), slightly higher, but not significantly different from values estimated for rabbits older than 5 weeks first challenged with the same virus (Student´s *t*-test = -1.31; df = 55; p = 0.195).

For factors associated with time to death, no model could be adjusted to data for the rabbits first challenged with GI.2 (n = 15). For rabbits first challenged with GI.1b, only the batch was retained because of the longer time to death in younger age classes (R^2 = 84.89%; n = 20; F = 47.77; p < 0.001). The model fitted to rabbits that were secondly challenged with GI.1b only retained ELISA absorbance values (R^2 = 26%; n = 22; F =

7.02; p = 0.010), indicating a direct relationship between this variable and time to death (parameter ± SE: 62.05 ± 23.41; *t* = 2.65; p = 0.010).

3.4. Duplex qPCR results

For all rabbits that died from RHD, duplex qPCR of liver tissue confirmed the presence of the virus used in the last challenge, with mean Cq values (± SD) of 18.50 (± 2.78) and 17.12 (± 2.21) for rabbits that died from GI.2 and GI.1b respectively. Analysis detected no coexistence of both viruses in the same sample for any case.

Rabbits euthanized after surviving both challenges (*n* = 42) were positive on the duplex qPCR in 30 cases (Table 4). The combined positivity rates for each virus (virus detected alone or in combination with another in the same sample) were directly correlated with rabbit age for GI.1b at the first challenge (Spearman *r* = 0.97; p = 0.005); however, this relationship was not evident for GI.2 or at the second challenge. The combined positivity rate for GI.2 was higher when this virus was used in the first challenge (0.65) than in the second (0.36) (two-tailed difference between proportions test p = 0.075); however, they were similar for GI.1b (0.52 *vs* 0.53; two-tailed difference between proportions test p = 0.955) (Table 4). Combined positivity rates for both viruses at the first challenge were not statistically significant (0.65 vs 0.52, respectively; two-tailed difference between proportions test p = 0.419), being similar to the positivity rate of GI.2 (4 from 8) for surviving rabbits that were only first challenged with this virus.

Positivity for both viruses occurred simultaneously in 12 of the 30 positive rabbits. Positivity rates for both viruses were higher in rabbits first infected with GI.2 (0.41) than in those first infected with GI.1b (0.20), mainly because most GI.1b-positive rabbits at the second challenge were also GI.2-positive (7 from 9), although not significantly (two-tailed Fisher´s exact test probability = 0.335). Positivity to both

viruses in rabbits first challenged with GI.1b was more balanced and did not differ significantly (two-tailed Fisher´s exact test probability = 1).

As expected, Cq values were higher in survivor rabbits than in RHD-dead rabbits. Mean Cq (± SD) values being 33.82 (± 4.90) and 33.48 (± 5.43) for GI.2 and GI.1b respectively.

4. Discussion

First reports conducted in France stated that GI.2 induces a more prolonged disease and a higher occurrence of subacute/chronic forms than GI.1 viruses (Le Gall-Reculé et al., 2013). In the present study, however, both isolates caused acute forms of RHD, and the average time to death post-infection was analogous, except for rabbits younger than 8 weeks infected with GI.1b. Clinical symptoms and lesions were also similar, including an increased spleen size, which depended on time to death post-infection but not on the virus type. Our results demonstrate that both viruses cause a similar disease, consistent with other surveys that reported the predominance of acute RHD forms caused by GI.2 (Dalton et al., 2012; Capucci et al., 2017; Carvalho et al., 2017).

After the first challenge, all surviving rabbits showed a clear seroconversion, confirming that all individuals were infected. Previous experimental challenges performed with GI.1b have shown that variations in haematological parameters occur a few hours after infection, with major changes peaking between 24 and 72 h post-infection, whereas surviving rabbits quickly recover normal values, usually before the 7th day (Ferreira et al., 2004; Ferreira et al., 2006a; Marques et al., 2012). Serum transaminase activity appears to follow a similar pattern (Marques et al., 2012), although Ferreira et al. (2004) reported persistently elevated AST and ALT values in young surviving rabbits through the 21st day post-infection.

In the present study, no marked deviations in blood parameters were observed compared with control rabbits from the 7th to 35th days post-infection, regardless of virus or rabbit age. This suggested that after the acute phase, the infection course was similar for both viral genotypes with no long-term changes; thus, even rabbits under 4 weeks old did not continue to undergo remarkable hepatic lesions.

Young rabbits are innately resistant to fatal infection by GI.1b (Marcato et al., 1991; Shien et al 2000; Ferreira et al., 2004). Marques et al. (2014) demonstrated that this protection can be reversed by artificial immunosuppression, suggesting that the innate immune mechanisms of young rabbits cause this resistance. Young RHD-dead rabbits also developed an acute form of the disease because GI.1b pathogenicity in rabbit kits is linked to high viral replication levels (Matthaei et al., 2014).

In our study, the GI.1b mortality pattern in young rabbits was unusual and implied an unplanned deviation from the initial experimental design, since the outstanding mortality rate registered for rabbits under 4 weeks old (21 of 25) has never been reported. These rabbits also developed an acute form of RHD but the mean time to death was considerably higher (double) than described in both adult and young rabbits (Marques et al., 2014; Matthaei et al., 2014).

Genetic background of the rabbit strain used could be an underlying putative cause since variations in *HGGA*, *SEC1* and *FUT2* genes have been associated with genetic susceptibility to the GI.1 virus (Guillon et al., 2009; Nyström et al. 2011). Another cause could be increased virulence as an adaptation to persist in high density wild rabbit populations (Elsworth et al., 2014). When we isolated the GI.1b virus, neither changes in RHD epidemiology nor major variations in the dynamic population were noticed in the wild rabbit population where the virus was circulating. Nevertheless, it is probable

that these changes were unnoticed because the rabbit population was at high density. This would imply that mean age at first infection was low, enhancing the role of kit protection by maternal antibodies and limiting RHD's negative impact at population level (Calvete 2006).

A third hypothesis could be immunosuppression induced by stress subsequent to early weaning, housing and management of the kits. However this hypothesis contrasts with the fact that no abnormal mortality pattern was observed in the batch of kits inoculated with GI.2, subjected to the same factors stress.

The GI.1b isolate was obtained two years before the occurrence of the new GI.2 that infects young rabbits. Recombination plays an important role in generating diversity in lagoviruses (Lopes et al., 2015; 2017). Although the *VP60* and *RdRp* gene sequences of our isolate corresponds to GI.1b genotype, full genomic analysis must be performed, and future studies are needed to identify the causes of these results.

As expected, mortality from GI.2 at the first challenge was lower than mortality by GI.1b, but the GI.2 mortality pattern was unrelated to rabbit age. Le Gall-Reculé et al. (2013) reported that pathogenicity differed significantly among GI.2 isolates and within them, to a lesser extent. In the present survey, GI.2 mortality rates varied considerably among rabbit batches (from 0 to 75%), demonstrating highly variable pathogenicity within the same isolate despite using rabbits from a single genetic strain and origin. This suggests that future studies to project GI.2 pathogenicity should consider this variability and that the differences among isolates should be interpreted with caution when small-scale assays are performed.

The factors associated with this variability are unknown. Our results showed that over 23% of the mortality rate variability caused by GI.2 at the first challenge was related to

variations in haematological parameters such as MCH, MCHC and lymphocyte and monocyte counts, whereas at the second challenge, 40% of the variability was associated with monocyte count. These haematological parameters are related to the three lines by which pluripotent bone marrow stem cells differentiate, the line of erythrocytes and blood platelets and the myeloid and lymphoid cell lines. Therefore, our results would indicate that individual innate differences in the immune system caused the susceptibility to developing a fatal disease after GI.2 infection. This result contrasts with that for GI.1b, for which only age was associated with mortality. This suggests that host-virus interaction mechanisms differ between both viruses and that GI.2 pathogenicity may be more modulated by innate immunity than GI.1b, which is mainly conditioned by obscure age-related factors.

An alternative hypothesis for this variable pathogenicity would be based on the small-scale assay of Capucci et al. (2017). These authors observed that the replication of highly pathogenic RHDV was strongly inhibited in rabbits suffering from severe enteritis (likely caused by enteric bacterial infection), suggesting that the prompt activation of the innate immune system by other pathogens could indirectly limit viral replication. Following their hypothesis, and given that our rabbits were healthy and no clinical signs were observed in any rabbits, our results may indicate that undetected concomitant infections by non-pathogenic agents could have occurred in some rabbits, determining their fate after the viral challenge. The mean haematological parameter values, however, were within baseline ranges determined by the rabbit supplier (data unpublished). Therefore, this hypothesis would not be strongly supported, although it can not be completely rejected either.

Cross-protection between both virus genotypes has been scarcely characterized. Mortality rates caused by GI.2 in rabbits vaccinated against GI.1b indicate limited

antibody roles in cross-protection (Le Gall-Reculé et al., 2011; Dalton et al., 2014). Moreover, in a small-scale assay, Le Gall-Reculé et al. (2013) showed that previous infection with GI.2 induced weak partial protection against GI.1b, whereas Peacock et al. (2017) confirmed that wild rabbits with naturally acquired anti-GI.1b antibodies can be infected by and die from GI.2.

In the present study, ELISA absorbance values were uncorrelated with mortality, supporting a minor role of antibodies in cross-resistance. Only longer time to death in rabbits that were secondly challenged with GI.1b was associated with higher absorbance values, likely because humoral immunity delayed productive viral replication. However, given the characteristics and limitations of the ELISA, it is impossible elucidate if rabbits that survived the second challenge had effective contact with the second virus and the nature of the immune response (if any) to the second challenge, either as a primary or secondary response; therefore, its role in cross-protection remains unknown.

Mortality rates caused by each virus were lower at the second challenge, confirming that partial, but asymmetric, cross-protection occurred. Strive et al. (2013) described a similar scenario where the non-pathogenic Australian rabbit calicivirus (RCV-A1) provided temporal and partial cross protection to GI.1c, unrelated to serum antibodies titres. These authors argued cellular immune mechanisms were the subjacent cause, since short-term elevation of non-specific innate immune mechanisms following the first infection may increase infectious disease resistance. This may be the reason for the asymmetric cross-protection observed in the present study. Given that host-virus interactions appear to differ between both viruses, and GI.2 pathogenicity may be modulated by innate immunity more than GI.1b, activation of innate immune mechanisms after the first challenge would yield a lower GI.2 pathogenicity at the second challenge.

Interestingly, this modulatory mechanism would imply that GI.2 interacts with other pathogens in both domestic and wild rabbits. Thus, other co-circulating microparasites (Myxoma viruses or other lagoviruses) could modulate the impact of RHD caused by GI.2 depending on outbreak timing or whether GI.2 was the first or second challenge for rabbits (Cox, 2001). Moreover, mortality caused by GI.1b at the second challenge was even higher than that caused by GI.2 at the first infection. This result contrasts with the effective replacement of GI.1b by the new viral genotype a few months after its spread (Le Gall-Reculé et al., 2013; Dalton et al., 2014; Calvete et al., 2014). Although GI.2's ability to infect kits implies a viral epidemiological advantage, the limited cross-protection and high mortality caused by GI.1b at the second challenge suggests that GI.1b viruses should have had greater probabilities to continue circulating in wild rabbit populations. However, interactions among lagoviruses, both at population and host levels are complex and poorly known (Strive et al., 2010; Strive et al., 2013). Therefore, because our results are a single estimate of cross-protection at a fixed time point (35 days after first infection), they are limited, and their implications in RHD epidemiology should be interpreted with caution.

Regarding duplex qPCR results in surviving rabbits, lagovirus RNA can be detected in convalescent rabbit tissues months after infection. The nature of lagoviral RNA persistence is unclear, and although this has led to the conclusion that persistent infections have been established, this RNA has not shown to be related to existing infective viruses, likely because the high antibody levels in the surviving animals originate immunocomplexes with viral particles (Shien et al., 2000; Forrester et al., 2003; Gall et al., 2007; Strive et al., 2010).

In the present study, viral RNA was detectable 10 weeks after infection at a similar rate (more than half the rabbits) for both viruses, but with some differences since GI.2

positivity rates showed no age-related pattern at the first challenge. The GI.2 positivity rates in rabbits that were secondly challenged were lower than those in the first challenge. This finding may suggest a more modulated GI.2 pathogenicity by a previous GI.1b infection, which may have lowered virulence or stimulated quicker GI.2 clearance.

All GI.1b-positive rabbits infected with this virus at the first challenge were younger than 8 weeks, and the positivity rate was directly correlated with age. These results agree with the hypothesis that young rabbits may be long-term GI.1b transmission vehicles (Ferreira et al., 2004), which is unlikely in older rabbits given their high mortality when infected with this viral genotype. GI.1b RNA was only detected in older rabbits when they were secondly challenged with this virus. In these rabbits, the positivity rate was similar than in young rabbits first infected with GI.1b, although the shorter time elapsed after the infection in older rabbits could have implied higher viral clearance.

Finally, co-occurrence of both viruses in the same sample did not statistically differ from a random distribution, although the results may indicate that GI.1b RNA from the second challenges was detected at a higher probability if GI.2 RNA had been present. However, these results are weak and should be carefully interpreted. For example, because RNA from both viruses was not detected simultaneously in any rabbit dead from RHD in the second challenge (27 animals by GI.1b and 2 by GI.2), mortality after the second challenge may have only occurred in rabbits that did not carry the virus from the first challenge and, therefore, the proportion of viral RNA-positive surviving rabbits was lower than that estimated after the second challenge, especially for GI.2. However, for this interpretation, it should be assumed that duplex qPCR was sufficiently sensitive

to detect very low viral quantities in tissues from rabbits that died from acute RHD caused by another one (no inhibition interference), but this has not been evaluated.

In conclusion, the present study demonstrates that the form of RHD caused by both viruses was similar, although the results showed that their host interactions differed markedly. An asymmetric partial cross-protection between both isolates was also determined, as were different mortality patterns: GI.2 was age-independent, whereas GI.1b was clearly modulated by age, although unexpectedly. Mortality caused by GI.2 was highly variable and related to individual factors, although the mechanisms are unknown. The results obtained, especially those regarding cross-protection, do not satisfactorily explain the quick and effective replacement of GI.1b viruses by the new GI.2 in the wild; thus, more studies are necessary to elucidate the interactions among these lagoviruses.

Conflict of interest statement

Conflicts of interest: none.

Autorship

CC, MM and JHC designed the study, performed experimental challenges and necropsies and drafted the manuscript; JHC and MPS designed and carried out biomolecular viral analyses; AA. and MPJ contributed with histopathological analysis of samples and AJC and FM participated in the development of assays. All authors contributed to the writing of the manuscript, read and approved the final version.

Acknowledgements

The study was supported by a Research and Development collaboration project between Agrifood Research and Technology Centre of Aragon (CITA) and the Animal Health,

Food Safety and Public Health Management Department of TRAGSATEC (INMUNIZADOS 1316) as well as by the E-RTA2014-00009-00-00 project from the Spanish National Institute for Agricultural and Food Research and Technology (INIA).

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FIGURE CAPTIONS

Fig.1. Variation of spleen weight to body weight ratio corrected by age (partial residuals are presented) in relation to time to death (horizontal axis, in hours). Black triangles: rabbits dead by GI.2 in first or second challenge; white circles: rabbits dead by GI.1b in first or second challenge; crosses: non-infected control and infected surviving-euthanized rabbits.

Fig.2. Mean (CI95%) of absorbance (OD) values measured with ELISA test for detection of serum antibodies against RHD viruses along the assay. Circles: non-infected control rabbits; triangles: rabbits first challenged with GI.2; squares: rabbits first challenged with GI.1b. In horizontal axis days since the first challenge. Second challenge was performed at day 35.

Fig. 3. Mortality rates and CI95% estimated for each experimental batch of rabbits after the oral infection with GI.2 or GI.1b viruses at first or second challenge. Vertical axe: mortality rate. Horizontal axe: age of rabbits (in weeks) in each experimental batch.

Fig.4. Mean time to death (in hours) and CI95% of rabbits dead by RHD in each experimental batch after the oral infection with GI.2 or GI.1b viruses at first or second challenge. Vertical axe: time in hours. Horizontal axe: age of rabbits (in weeks) in each experimental batch.

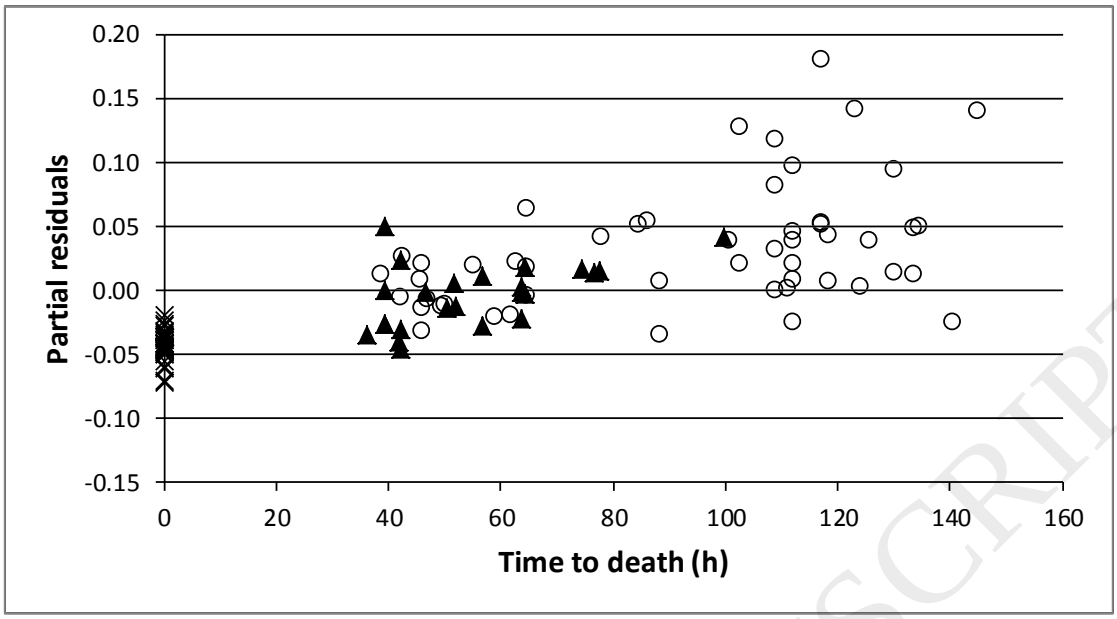

Fig.1.

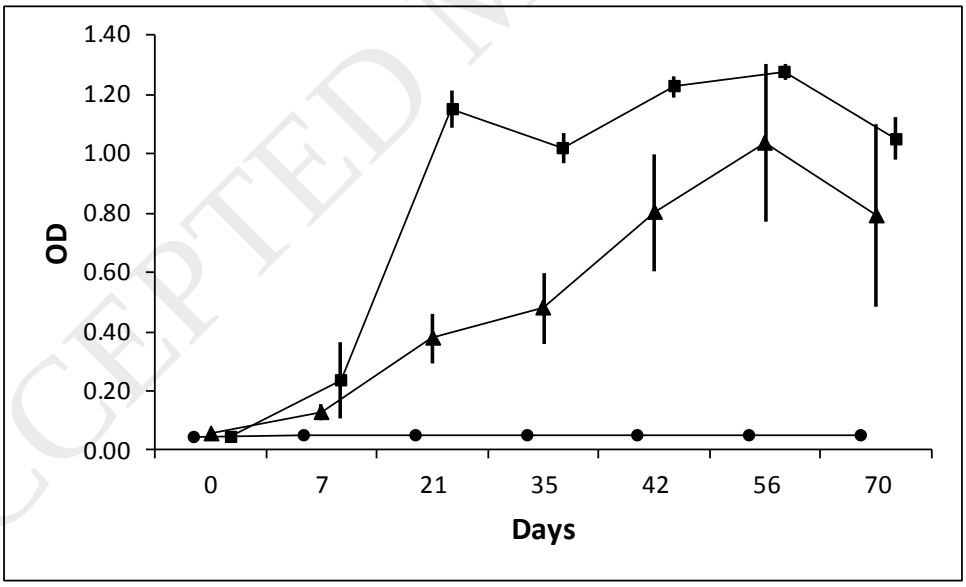

Fig.2.

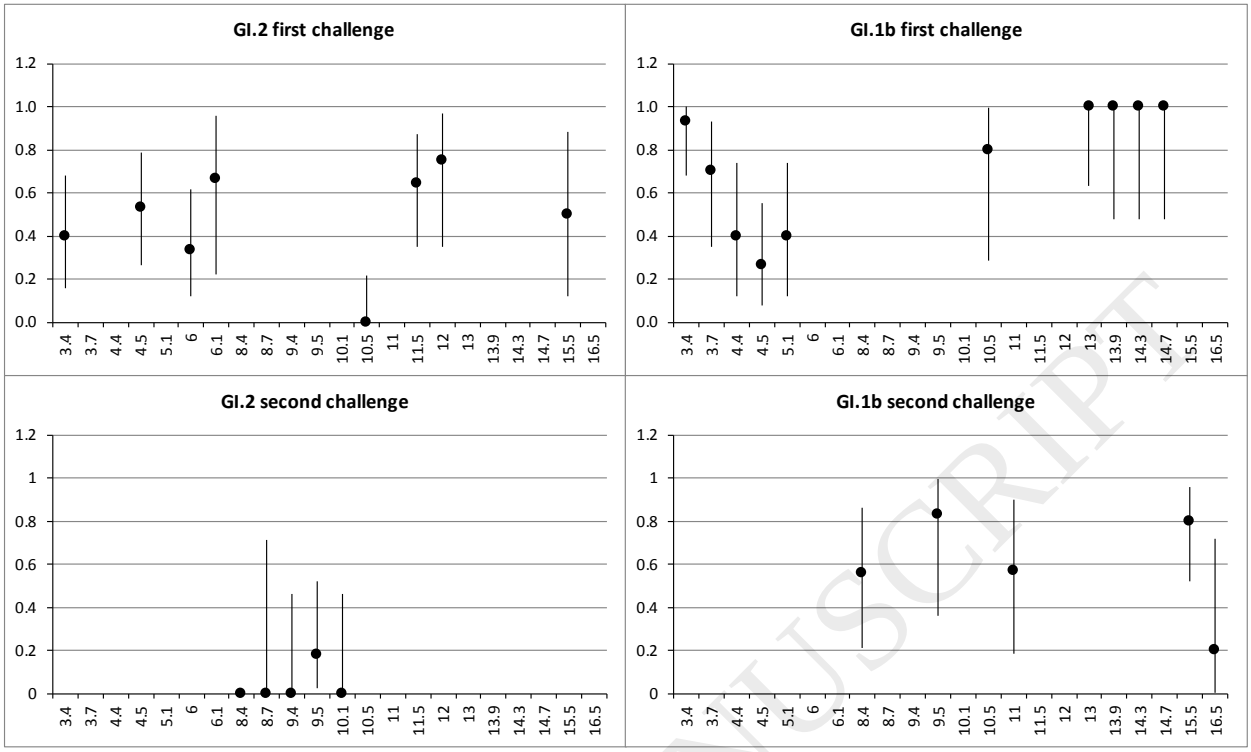

Fig. 3.

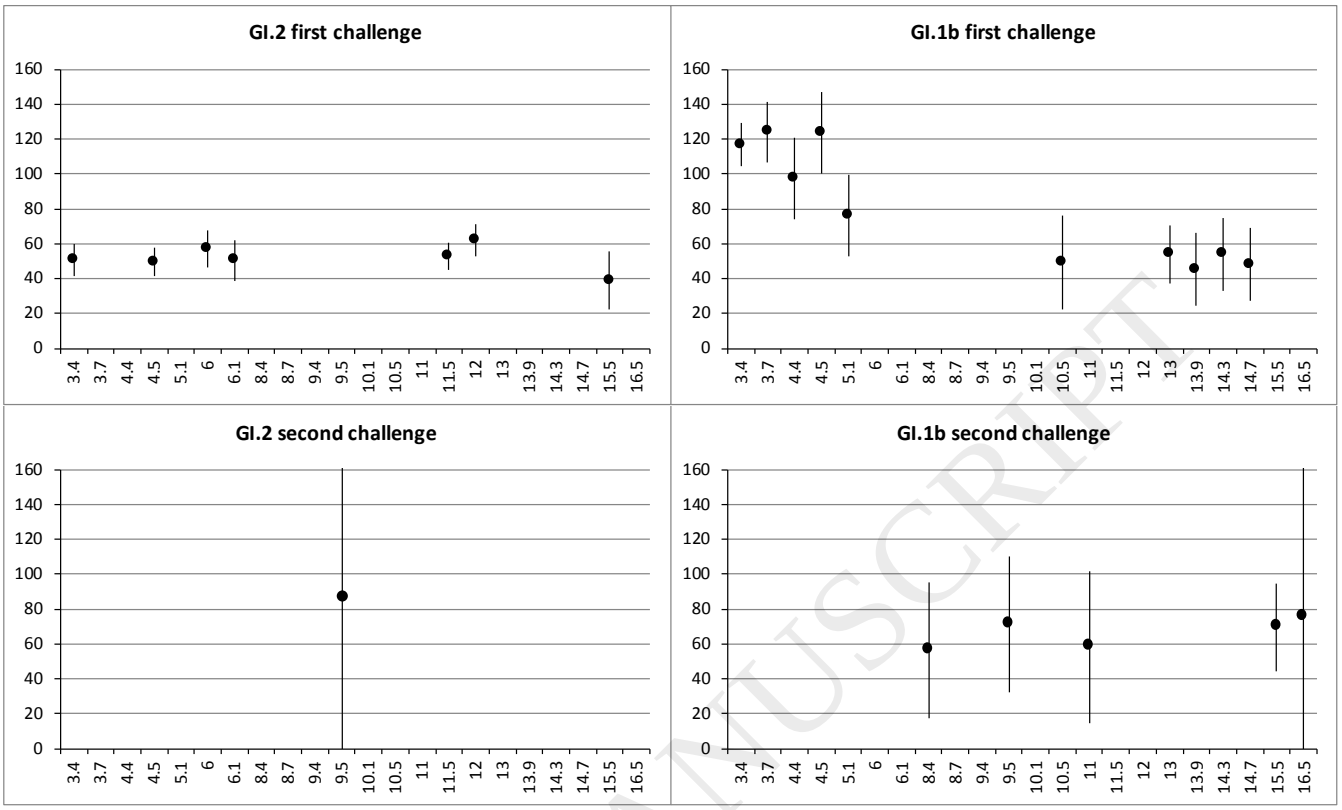

Fig.4.

Table 1

Challenged rabbits distribution across age-batches and virus isolates (RHD-dead rabbits/challenged rabbits). Second challenge with a virus was performed on rabbits surviving the first challenge by the other virus.

First challenge			Second challenge		
Age (weeks)	GI.2	GI.1b	Age (weeks)	GI.1b	GI.2
3.4	6/15	14/15	8.4	5/9	0/1
3.7		7/10	8.7		0/3
4.4		4/10	9.4		0/6
4.5	8/15	4/15	9.5	5/6	2/11
5.1		4/10	10.1		0/6
6.0	5/15		11.0	4/9	
6.1	4/6*				
10.5	0/15	3/5**	15.5	12/15	
11.5	9/14		16.5	1/5	
12.0	6/8*				
13.0		8/8***			
13.9		5/5***			
14.3		5/5***			
14.7		5/5***			

* Surviving rabbits were not secondly challenged with GI.1b.

** The two surviving rabbits euthanized after developing chronic RHD form.

*** Added batches

Table 2

Poisson regression final models fitted to mortality data of rabbits infected with GI.2 at first and second challenges.

	Mortality rates ratio (SE)	*z*	p
First challenge model			
MCH (pg)	2.211 (0.465)	3.77	<0.001
MCHC (g/dl)	0.435 (0.091)	-3.99	<0.001
Lymphocyte / mm^3	1.001 (0.0003)	2.38	0.01
Monocyte / mm^3	0.997 (0.001)	-2.61	0.009
	Pseudo-R^2 = 22.82%; n = 49; Wald χ^2 =25.69; p < 0.001		
Second challenge model			
Monocyte / mm^3	0.987 (0.004)	-3.45	0.001
	Pseudo-R^2 = 40.22%; n = 25; Wald χ^2 =11.93; p < 0.001		

Table 3

Observed mean (SD) values for each experimental age-batch of haematological parameters retained in the final Poisson regression model fitted to mortality of rabbits infected at the first challenge with GI.2.

Age of batch	3.4 weeks	4.5 weeks	10.5 weeks	15.5 weeks
n	13	15	15	6
MCHC (g/dl)	29.05	29.54	31.08	31.26
(SD)	(3.41)	(2.44)	(0.57)	(0.47)
MCH (pg)	21.42	21.50	20.35	20.33
(SD)	(3.09)	(1.67)	(0.72)	(0.73)
Lymphocyte / mm^3	1764.96	2115.79	4343.37	5232.94
(SD)	(508.53)	(443.86)	(1229.99)	(1216.93)
Monocyte / mm^3	429.87	570.20	814.18	677.44
(SD)	(177.25)	(147.63)	(345.49)	(223.46)

Table 4

Positive duplex qPCR results in liver tissues from rabbits surviving to both challenges (positivity rates in brackets). Combined results include positive results to one virus plus positive results to both viruses.

Rabbits first challenged with GI.2

Age*	GI.2	Both**	GI.1b	GI.2 combined	GI.1b combined	*n*
3.4	2 (0.50)	0 (0)	0 (0)	2 (0.50)	0 (0)	4
4.5	0 (0)	1 (1)	0 (0)	1 (1)	1 (1)	1
6.0	1 (0.20)	4 (0.80)	0 (0)	5 (1)	4 (0.80)	5
10.5	0 (0)	0 (0)	2 (0.67)	0 (0)	2 (0.67)	3
11.5	1 (0.25)	2 (0.50)	0 (0)	3 (0.75)	2 (0.50)	4
Total	4 (0.23)	7 (0.41)	2 (0.12)	11 (0.65)	9 (0.53)	17

Rabbits first challenged with GI.1b

Age*	GI.1b	Both**	GI.2	GI.1b combined	GI.2 combined	*n*
3.4	0 (0)	0 (0)	1 (1)	0 (0)	1 (1)	1
3.7	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	3
4.4	2 (0.33)	0 (0)	0 (0)	2 (0.33)	0 (0)	6
4.5	1 (0.11)	5 (0.55)	3 (0.33)	6 (0.67)	8 (0.89)	9
5.1	5 (0.83)	0 (0)	0 (0)	5 (0.83)	0 (0)	6
Total	8 (0.32)	5 (0.20)	4 (0.16)	13 (0.52)	9 (0.36)	25

* Age at first challenge in weeks. Second challenge was carried out 5 weeks later.

** Duplex qPCR positive to both viruses simultaneously