World Rabbit Science

World Rabbit Sci. 2019, 27: 143-156
doi:10.4995/wrs.2019.11082

RABBIT HAEMORRHAGIC DISEASE: EXPERIMENTAL STUDY OF A RECENT HIGHLY PATHOGENIC GI.2/RHDV2/b STRAIN AND EVALUATION OF VACCINE EFFICACY

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Abstract: In 2010, a variant of the rabbit haemorrhagic disease virus (RHDV) belonging to a new GI.2 genotype was identified in France and rapidly spread worldwide. Due to antigenic difference, new vaccines including G1.2 strains have been developed to confer adequate protection. An increase in the pathogenicity of the circulating strains was recently reported. The objective of this experimental study was to characterise the infection with a highly pathogenic GI.2/RHDV2/b isolate (2017) and assess the efficacy of Filavac VHD K C+V vaccine (Filavie) against this strain. Four and 10-wk-old specific pathogen-free rabbits were inoculated with a recommended dose of vaccine. After 7 d, controls and vaccinated rabbits were challenged and clinically monitored for 14 d. All animals were necropsied and blood, organs and urine were sampled for quantitative reverse transcription polymerase chain reaction (RT-qPCR) analysis. In adult groups, regular nasal and rectal swabbing were performed, and faeces were collected after death to monitor RNA shedding. In control groups, the challenge strain induced acute RHD between 31 and 72 h post-inoculation, with a mortality rate of 100% for kits and 89% for adult rabbits. Except for a shorter mean time to death in kits, similar clinical signs and lesions were observed between age groups. The vaccination significantly prevented all mortality, clinical signs, detection of viral RNA in serum and gross lesions in kits and adult rabbits. In adult groups, we also demonstrated that vaccine significantly protected from detectable RNA shedding via naso-conjunctival and rectal routes. Two weeks after challenge, RNA copies were not detected by PCR in the liver, spleen, lungs, kidneys, faeces and urine of vaccinated adult rabbits. The findings for kits were similar, except that very low levels of RNA were present in the liver and spleen of a few rabbits. These data show that immunisation prevented any significant viral multiplication and/or allowed a rapid clearance. We concluded that, despite the quick evolution of GI.2/RHDV2/b strains, the protection conferred by the vaccine remains adequate. In the context of coexistence of both GI.1 and GI.2 genotypes in some countries, with the circulation of multiples recombinant viruses, the vaccination should be based on the association of strains from both genotypes.

Key Words: rabbit haemorrhagic disease (RHD), GI.2/RHDV2/b, pathogenicity, vaccine, efficacy, RT-qPCR.

INTRODUCTION

Rabbit haemorrhagic disease (RHD) is a highly contagious and usually acute disease of wild and domestic European rabbits (*Oryctolagus cuniculus*) (reviewed in Abrantes *et al.,* 2012). The aetiological agents, known as rabbit haemorrhagic disease virus (RHDV), are small non-enveloped single-stranded RNA viruses belonging to the species *Lagovirus europaeus,* within the family *Caliciviridae* (according to new nomenclature proposed by Le Pendu *et al.,* 2017). First reported in China in 1984, the disease has rapidly spread worldwide and is currently considered endemic in many European countries, Australia and New Zealand. The disease is characterised by acute necrotising

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https://doi.org/10.4995/wrs.2019.11082

hepatitis, generally associated with disseminated intravascular coagulation leading to haemorrhages in many organs (particularly lungs and kidneys). The first isolates of RHDV –previously qualified as "classic", and currently designated GI.1 genotype (Le Pendu *et al.,* 2017)– induced high mortality rates (70-100%) in rabbits older than 10 wk of age, while kits 4-6 wk of age were innately resistant to fatal infection (reviewed in OIE, 2017). The initial RHD epidemic strongly affected wild populations and the rabbit industry. In addition to strict biosecurity measures, the development of efficient vaccines afforded control of the disease in rabbitries in the 90s.

In 2010, a new virus, currently referred to as RHDV2/b (Le Pendu *et al.,* 2017), was identified in France following atypical outbreaks of RHD in kits (15-25 d old) and in adult vaccinated rabbits (Le Gall-Reculé *et al.,* 2011, 2013). This virus induced lower (and more variable) mortality in adult rabbits than GI.1/RHDV viruses, with an average mortality rate of 30% recorded after natural infections and experimental challenges. Chronic and subacute forms were more frequent (Le Gall-Reculé *et al.,* 2013). Kits (<5-wk-old), so far highly resistant to classical RHD caused by GI.1 viruses, succumbed to the infection with the RHDV2/b (Dalton *et al.,* 2012). As suggested by initial field data (Le Gall-Reculé *et al.,* 2011; Dalton *et al.,* 2012), experimental studies confirmed that rabbits vaccinated against GI.1/RHDV were not protected against challenge with RHDV2/b, and vice-versa (absence of cross-protection) (Le Minor *et al.,* 2013; and unpublished data). The antigenic profile indicated clear differences with GI.1 strains in the viral surface where neutralising epitopes are located (Le Gall-Reculé *et al.,* 2013). Sequencing of VP60 gene revealed that it constitutes a new phylogenetic group (Le Gall-Reculé *et al.,* 2013).

This virus, with distinct pathogenic, genetic and antigenic profiles, was included in a new genotype, GI.2 (Le Pendu *et al.,* 2017). It rapidly spread to other European countries, causing severe outbreaks in rabbit farms and in wild populations (Abrantes *et al.,* 2013; Le Gall-Reculé *et al.,* 2013; Dalton *et al.,* 2014; Westcott *et al.,* 2014; Duarte *et al.,* 2015), and was later detected in Australia, Northern America and Africa (Hall *et al.,* 2015; Martin-Alonso *et al.,* 2016; OIE, 2016). In some European countries and in Australia, GI.2/RHDV2/b viruses are supplanting endemic GI.1/RHDV strains, although the mechanisms underlying this replacement are yet unknown (Le Gall-Reculé *et al.,* 2013; Calvete *et al.,* 2014; Dalton *et al.,* 2014; Mahar *et al.,* 2018). In addition, the new genotype exhibits a larger host range than older strains, by sporadically infecting different hare species (Puggioni *et al.,* 2013; Le Gall-Reculé *et al.,* 2017). Recombination event seems to play a major role in the quick evolution of the new variant, as multiple recombinant viruses have been reported in Europe and Australia (Lopes *et al.,* 2015; Hall *et al.,* 2015; Silverio *et al.,* 2018).

In 2015, a new inactivated polyvalent vaccine (Filavac VHD K C+V; Filavie) containing strains of each genotype GI.1/RHDV and GI.2/RHDV2/b became available in France. Its efficacy against GI.1 and GI.2 challenges has been demonstrated from 1 wk (onset) up to 18 mo (duration of immunity) in specific pathogen-free (SPF) rabbits vaccinated at 11 wk of age (Morin *et al.,* 2015). Sins 2016, more fatal GI.2/RHDV2/b infections have been reported in very young kits, so the vaccination of kits before weaning became a crucial issue. Le Minor *et al.* (2017) demonstrated that, in experimental conditions, a vaccination at 2 and 3 wk of age was safe and conferred a full and rapid GI.2/RHDV2/b protection.

Recently, a worrying increase in the severity of RHD cases has been observed, both in wildlife and livestock. Several GI.2/RHDV2/b outbreaks in unvaccinated farmed rabbits were characterised by higher mortality rates (Capucci *et al.,* 2017). Experimental studies of GI.2 strains isolated in 2014 and 2015 in Italy, as well as recombinant GI.1bP-GI.2 strain isolated in Australia in 2015, confirmed that these recent viruses resulted predominantly in an acute disease unlike the earliest emerging strains (2010-2011), which often induced subacute and chronic form of RHD (Capucci *et al.,* 2017; Neimanis *et al.,* 2018). Mortality rates of at least 80% were recorded, which approached usual mortality rate described for GI.1/RHDV, and demonstrated an increase in pathogenicity of GI.2/RHDV2/b viruses.

In the current context of rapid GI.2/RHDV2/b evolution, with a significant increase in pathogenicity, and also considering the possibility of a new antigenic slip, it is essential to assess the efficacy of commercial vaccines against current circulating GI.2/RHDV2/b strains. In addition, there is a need to better characterise viral infection with highly pathogenic GI.2/RHDV2/b, as very few experimental data have been published. Therefore, the aim of the present study was to assess, under experimental conditions, the level of protection conferred by Filavac VHD K C+V vaccine against a recent highly pathogenic GI.2/RHDV2/b isolate, and at the same time, to collect information in order to describe the clinical course, virulence and viral RNA distribution (and persistence) in organs and excreta of infected

animals. In addition to 10-wk-old rabbits, which corresponds to the minimum age recommended for this vaccine, the experiment also included kits at weaning, as this period represents a major issue in the control of the disease under field conditions.

MATERIAL AND METHODS

Experimental animals and ethical considerations

SPF New Zealand White rabbits, males and females, were purchased from an authorised rabbit supplier. Twenty 4-wk-old rabbits, weighing 410-670 g, and the same number of 10-wk-old rabbits, weighing 1.8-2.5 kg, identified by an individual number, were included in the study. They were housed in groups of 5 animals in ventilated isolators, with free access to water and food, and kept under observation during a 24-h-acclimatisation period. The ambient temperature inside the isolators was kept between 20 and 23°C, with a light/dark cycle of 12 h.

Experimental infections were performed under negative pressure in BLS2 experimental facilities with filtered air. Animal experiments were approved by the French Ministry of Research (protocol APAFIS 3081) and conducted according to the requirements of national (2013-118 decree) and European (2010/63/EU) laws on the handling of animals used for scientific purposes.

Experimental design

The rabbits from each age group were randomly allocated to 2 groups of 10 animals: one was vaccinated on day 0 (D0), whereas the other group was kept as control. Vaccinates (V4 and V10 groups) and controls (C4 and C10 groups) were challenged on day 7 (D7) and observed over a 14-d-period (until D21). All surviving rabbits on D21, as well as all animals reaching ethical endpoints during the experiment, were euthanised by intravenous injection of 182.2 mg/kg of sodium pentobarbital (Doléthal®, Vétoquinol) in the marginal ear vein. Specific RHD clinical endpoints were defined as: lethargy (unable to avoid capture), lateral recumbency, anorexia, dyspnoea, vocalisations and terminal neurological signs (paddling, convulsion, etc.).

Vaccine

Filavac K C+V® vaccine (Filavie), containing inactivated GI.1/RHDV and GI.2/RHDV2/b lagoviruses (isolated in French rabbitries, respectively in 2011 and 2012), was used. Following the manufacturer's instructions, rabbits were inoculated subcutaneously with a recommended dose of 0.2 mL.

Challenge strain and challenge protocol

GI.2-OLM.2017 strain was collected in October 2017 in a rabbitry located in the western part of France where an acute outbreak of RHD was occurring. The strain induced a very high mortality rate (80%) in unvaccinated kits. The virus was passed onto SPF kits and a liver supernatant (viral seed) was prepared from the liver of one animal that died of acute RHD. The challenge inoculum was prepared according to the same protocol and dilution of the viral seed as those used for a challenge with an older GI.2 strain ("CH5801.SV" thereafter GI.2-CH.2012, please refer to Discussion section). Pathogenicity of this inoculum was confirmed in SPF kits during preparatory experiments. The inoculum was administered by intramuscular injection.

Clinical and post-mortem examination

Rabbits were observed daily from D0 to D21 for their general health status. Each animal was weighted on D0 and D21. Between D8 to D11 (i.e., 1 to 4 d post-challenge [dpc]), the period previously identified as having the highest RHD mortality risk, animals were monitored at least 3 times daily in order to identify ethical endpoints as soon as possible. Rectal temperature was measured just before challenge (on D7), after 24 h (on D8) and weekly (D15, D21) for surviving animals. Temperature was also recorded before ethical euthanasia. Normal rectal temperature is defined as 38.5-40°C.

During the study, animals found dead or humanely euthanised, as well as surviving animals euthanised at the end of the experiment, were necropsied and examined for macroscopic lesions.

Blood, organs, urine, faeces and swabs collection

Two rabbits in each group were randomly selected and sampled on D0 to confirm seronegativity against GI.1/RHDV. Then, blood samples were taken on D8, D15 and D21 from 5 (if possible) surviving rabbits in each group (if more than 5 animals survived, rabbits were selected at random), and just after euthanasia (human endpoints). Due to the difficulty of collecting blood from 5-wk-old animals, only 2 kits per group were sampled on D8. Blood was collected in a citrate vacutainer using the marginal ear vein or by cardiac puncture following euthanasia. Serum was obtained after centrifugation at 20°C for 5 min at 3400 rpm and stored at –70°C until serological and quantitative reverse transcription polymerase chain reaction (RT-qPCR) analysis.

To study viral RNA shedding, dry naso-conjunctival and rectal swabs were collected from all (surviving) adults on D8, D10, D15, D21 and all adults that succumbed to the disease. Each swab was immersed in 200 µL of pure water and vortexed twice for 1 min. Then the swab was wrung out and the liquid was stored at –70°C until molecular analysis.

After euthanasia or death, the liver was sampled in all animals. Spleen, kidney, lung, urine (and faeces for adult groups) were collected in at least 3 animals per group. All tissue samples were homogenised with phosphate buffered saline (PBS) and clarified at 2500 rpm for 30 min. The supernatants, urines and faeces were stored at –70°C.

Detection of GI.1 specific antibodies by ELISA

Due to the absence of a commercial test for GI.2/RHDV2/b, only the detection of antibodies as GI.1/RHDV was carried out in sera using an indirect ELISA test (Ingezim Rabbit, INGENASA Lab., Madrid, Spain). This test enabled us to check the seroconversion due to the immunisation with the polyvalent vaccine.

Histopathological examination

Six rabbits, from both age groups, with typical gross lesions, were selected for histological analysis. Analysis examined lymph nodes, thymus, trachea, salivary gland, thyroid, lung, liver, kidney, spleen, duodenum, jejunum and pancreas specimens (eyes were also sampled in one C10 animal). Samples were fixed in 4% neutral buffered formalin, embedded in paraffin, sectioned at 3 µm, mounted on glass slide and stained with haematoxylin and eosin. One slide per animal was prepared, except for one rabbit, for which the 2 eyes were included in a second slide. The slides were blindly analysed under a light microscope Nikon Eclipse Ci.

Quantification of RHDV2/b RNA by real-time RT-PCR

Total RNA was extracted from 150 µL of organ supernatants diluted to the twentieth in PBS, 100 µL of undiluted serum and 150 µL of undiluted urine using a Nucleospin RNA virus kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions and eluted in 50 µL of DNase/RNase-free water. Total RNA was extracted from 30 mg of faeces using Nucleospin RNA stool kit (Macherey-Nagel, Düren, Germany) following the manufacturer's instructions for rabbit stool and eluted in 100 µL of DNase/RNase-free water.

The primers and probe of the RHDV2 RT-qPCR were designed by a private laboratory (Scanelis, Toulouse, France) based on conserved regions evidenced in the alignment of VP60 complete RHDV2 sequences. The RT-qPCR was performed using an ABI Prism 7500 Fast platform (Applied Biosystem, Madrid, Spain) in a 96-well optical plate format. Amplification was carried out in 25 µL volume reactions containing 5 µL of RNA extract and 20 µL of Scanelis RHDV2 reaction mix. Thermal cycling conditions included one cycle at 50°C for 20 min for reverse transcription, one cycle at 95°C for 15 min for Taq polymerase activation and 50 cycles of cDNA amplification (95°C for 15 s and 60°C for 30 s). Fluorescence was acquired during each extension step. Negative controls contained PCR-grade water. All samples were amplified once. A range of 3 tenfold dilutions of a quantified transcribed RNA was added to the plate. Each dilution was amplified twice. The decimal logarithm of the RNA copy number of each dilution was plotted against its cycle threshold value (Cq value) in Excel®. Slope and Y-intercept of the linear regression were determined to be used for the calculation of the RNA copy number/5 µL of samples. An equivalency between Cq values and decimal

logarithm of RNA copy number was established. The RNA copy number/5 µL of the samples was calculated using the following formula:

$$\text{RNA copynumber/5µL} = 10^{\left(\frac{\text{Ct value}_{\text{sample}} - \text{Yintercept value}}{\text{Slope value}}\right)}$$

Then, different dilution factors were applied to convert the RNA quantity in copies/5 µL to RNA copies/mg of tissues. In liver matrix, the limit of detection of the RT-qPCR method was 2.3 $\log_{10}$ copies/mg (detection limit was not studied for other tissues).

Statistical analysis

Statistical analyses were carried out using the Stata® 10.0 for Windows software.

Two-sided tests were performed with a statistical significance threshold of 0.05 (5%). Mean values were compared using a non-parametric Mann-Whitney test (or Wilcoxon-Mann-Whitney). Percentages were compared with the Fisher's exact test.

RESULTS

Serological status and clinical observations after vaccination

On D0, prior to vaccination, all the rabbits sampled (2 per group) were confirmed to be free of antibody against GI.1/RHDV by ELISA, as expected considering their SPF status. Between D8 to D15, all the vaccinated animals sampled (5/5 kits and 5/5 adults) showed seroconversion, while control remained seronegative (3 kits and 5 adults were tested).

Vaccinated rabbits remained healthy and no adverse signs were recorded. One vaccinated and one control rabbits (V10 and C10) showed paralysis of the hind legs, probably due to inadequate new platforms set up in the isolators. They were ethically euthanised before challenge.

Clinical signs, mortality and time to death after challenge

Just before challenge, all individual rectal temperatures were included within the normal range for rabbits. Identical mean temperatures were recorded in both kit groups, (39.5±0.2°C [±standard deviation]) for C4 and 39.5±0.3°C for V4 [n = 10/group]), as well as in both adult groups (39.3±0.2°C for C10 and V10 [n = 9/group]). Twenty-four hours post-challenge, mean temperature increased significantly in C4 group (+0.9°C, $z = 2.13$, $P = 0.03$ for Mann-Whitney test). No significant increase was observed in mean temperature in V4 group, as well as in C10 and V10 groups (+0.2 in each group). Five control animals developed fever (41.5°C recorded for four C4 animals, 41°C in one C10 animal). After 7 additional hours, the temperature of these animals dropped (between –0.2 to –3°C). These rabbits were the first to succumb to the disease (dead or humanly euthanised between 31 to 45 h post-challenge). Individual rectal temperatures of vaccinated animals remained normal throughout the follow-up, with a mean temperature of 39.4°C (range: 39.0-39.7°C) and 39.2°C (38.8-39.4°C) for kits, and of 39°C (38.8-39.4°C) and 39.3°C (39.1-39.5°C) for adults, respectively 8 and 14 dpc.

High mortality rates were recorded among control groups, as all (10/10) the kits and 8 out of 9 adults died, or reached human endpoints (Figure 1). Estimated time to death ranged between 31 to 63 h for kit rabbits, and between 40 to 72 h for adult rabbits, with a mean time to death slightly higher for adults (58 h *vs.* 42 h, $z = -3.08$, $P = 0.002$ for MW test).

G1.2-OLM.2017 challenge caused similar signs in 5 and 11-wk-old rabbits. Typical signs of terminal RHD, considered as ethical endpoints, were observed in 5 control rabbits: marked depression (showing no resistance to capture), lateral recumbency and/or with the head down. In addition, some animals showed dyspnoea at a very late stage of the evolution. Interestingly, jaundice was one of the early signs almost constantly present. The yellowish coloration of the conjunctiva and skin (mostly visible on the inner side of the pinna) was associated with a light yellow urine with high amounts of pigments. A discrete nasal discharge (serous), resulting in a small yellowish deposit at the base of the

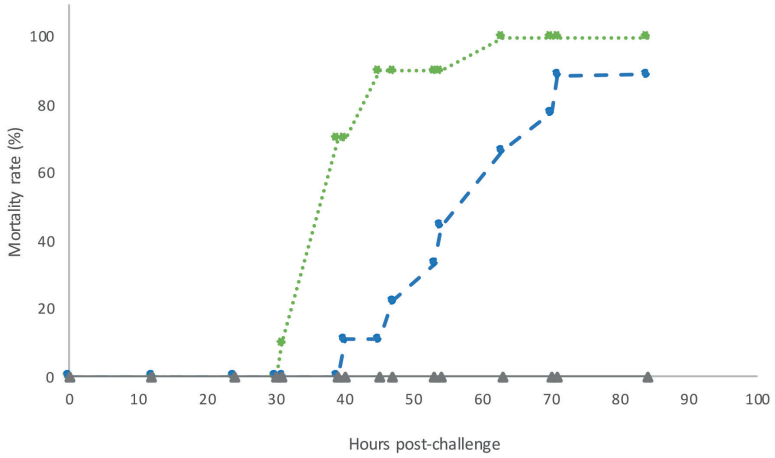


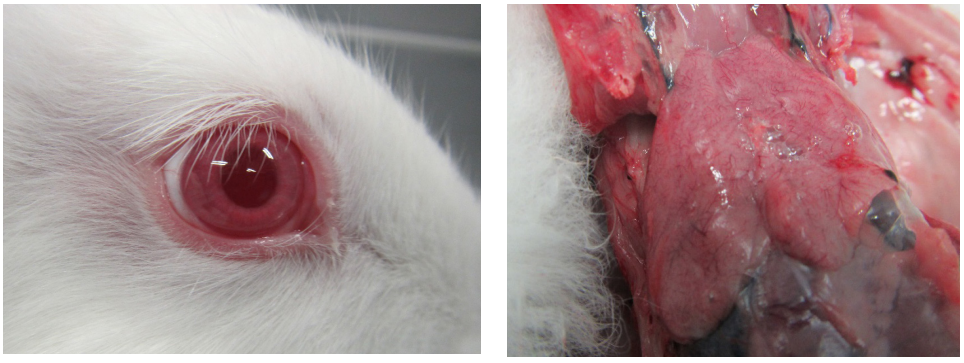
Figure 1: Evolution of the mortality rate per group until 5 d post-challenge. C10, 10-wk-old control rabbits; C4, 4-wk-old control rabbits; V10, 10-wk-old vaccinated rabbits; V4, 4-wk-old vaccinated rabbits. ···●··· C4, —●— C10, —▲— V4+V10.

nostrils, was observed in some rabbits. Frequently, a few hours before death, the eyes of the (albino) rabbits turned dark red (Figure 2-A). In contrast, epistaxis was recorded in only 2 (out of 18) affected control animals.

All the vaccinated animals and one control animal (C10 group) remained healthy throughout the experiment. Kits (V4) showed a regular body weight development in compliance with the species standard and sex (average daily gain±standard deviation) = 42±3.8 g between 4 and 7-wk-old). Survival rates were significantly higher in the vaccinated groups than in the respective control groups (100% *vs.* 10% or 0%, $P<0.0001$ for Fisher's exact test).

Gross pathology and histopathology

At necropsy, both jaundice and haemorrhagic lesions were present. Marked icteric lesions, which appeared earlier in the development of the disease, affected all the organs but were mostly visible on the liver, muscle, fat and conjunctiva. Haemorrhagic lesions were observed at the very end (probably a few hours before death) mostly in the lungs, kidneys and thymus. Histopathological lesions were of similar morphology in all affected animals (4 kits and 2 adults examined), regardless of the age of the animals. As expected, lesions were more severe in animals found



A B

Figure 2: Clinical signs and gross lesions in the organs of control rabbits after GI.2-OLM.2017 challenge. A) Highly congested eyes (dark red). B) Enlarged and congested thymus.

dead than in animals humanely euthanised a few hours before death. Major macroscopic and histological results are summarised in Table 1, and associated pictures are presented in Figure 2.

No gross lesions were found in any of the vaccinated rabbits and in the control rabbit that survived.

Table 1: Description of most typical lesions observed in the organs of control rabbits after GI.2-OLM.2017 challenge.

Organ	Macroscopic and microscopic observations
Liver	Macro: Enlarged, discoloured and icteric (yellow-tan). Micro: Multifocally, individual or aggregates of hepatocytes are necrotic, affecting preferentially periportal areas, including cells of the limiting plate, and in a lesser extend midzonal areas, associated with a mild infiltration by heterophils and fewer macrophages within the sinusoids and in the areas of necrosis. Necrotic hepatocytes have a shrunken, hypereosinophilic, often fragmented cytoplasm and a pyknotic or karyorrhectic nucleus. Adjacent hepatocytes are degenerated, characterised by a vacuolated cytoplasm and a lytic nucleus. Few acute haemorrhages are observed in areas of necrosis.
Spleen	Macro: Enlarged, purple to dark red coloration. Micro: The spleen is moderately congested. The white pulp is moderately depleted. The red pulp shows a moderate hypertrophy of endothelial cells associated with a hyaline aspect of the stroma and an increase of circulating activated macrophages sometimes showing erythrophagocytosis. Occasional nuclear fragments are dispersed in the red pulp.
Trachea	Macro: Congested, lumen filled with blood/bloody foam. Micro: The lamina propria is slightly congested.
Lungs	Macro: Fully congested, mottled dark red. Micro: The lung is mildly congested. The parenchyma is markedly congested and multifocal mild alveolar oedema is present. Rare fibrin thrombi are observed in alveolar capillaries associated with multifocal mild acute alveolar haemorrhages and the occasional presence of necrotic cells in alveolar septae. Few macrophages are observed in alveolar lumen.
Kidneys	Macro: Enlarged, dark red coloration. Haemorrhages in glomerular and renal medullary loops. Micro: Glomeruli are diffusely severely congested and multiple fibrin thrombi are present in glomerular capillaries, associated with endothelial and mesangial cell hypertrophy, lysis or rarely necrosis. The interstitium is diffusely moderately congested and contain multifocal mild acute haemorrhages. The tubules are slightly dilated with an attenuated epithelium.
Salivary glands	Macro: Congested. Micro: Slightly congested.
Duodenum Jejunum	Macro: Congested. Micro: Absence of lesion.
Pancreas	Macro: Congested. Micro: Absence of lesion.
Thymus	Macro: Enlarged with petechial haemorrhages. Micro: The thymus is slightly congested. The cortex shows a mild increased of tangible body macrophages without lymphoid depletion or lymphocytolysis. Rare interlobular acute haemorrhages are present.
Thyroid	Macro: (Highly) congested. Micro: The parenchyma is slightly congested.
Lymphatic nodes	Macro: Enlarged (without haemorrhages). Micro: Absence of lesion.
Eyes	Macro: Dark red coloration (haemorrhagic). Micro: The connective and the adipose tissue in periocular location are severely congested.
Skin /fat	Macro: Yellowish coloration (bile pigment accumulation)
Blood	Macro: Clotting defect and delay.

Detection of viral RNA in organs

All unvaccinated control animals which succumbed to the disease had a high level of RNA copies in their livers, spleens, kidneys and lungs (Figure 3). For liver and spleen, lower viral loads were obtained in animals which were humanly euthanised before death, which should have shortened the last phase of virus multiplication. In affected animals, the highest and most uniform level of genome concentration was found in the liver (range: 7.7 to 8.7 $\log_{10}$ copies/µg). The median copy number was higher in kits than in adults for most tissues (liver, spleen and kidney). This may be partly related to the higher number of euthanised animals in that group.

At the end of the study (14 dpc), a small residual amount of RNA (2.9 $\log_{10}$ copies/µg) was still present in the liver of the only surviving control rabbit (C10), which confirms that it was infected. Extremely low levels of RNA (1.8 to 2.2 $\log_{10}$ copies/µg, i.e. below the safe detection limit) were also detected in the liver of 3 out 10 vaccinated kits (10^6 to 10^7-times lower than in kits that succumbed to the infection). The spleen was the only other positive tissue in vaccinated kits (amount 10^4 to 10^6-times lower than in the control kits). All the organs of vaccinated adults were qPCR negative.

Detection of viral RNA in sera

Sera of all control rabbits sampled 1 dpc (2 kits and 5 adults) contained RNA genome, while none of the sera of vaccinated ones (same sampling) were PCR-positive (Table 2). When data from different age groups were pooled, vaccination had a significant effect on whether a rabbit was a positive or negative for virus detection in serum 1 dpc (detection rate: 100% in control *vs.* 0% in vaccinated rabbits, $P=0.001$ for Fisher's exact test).

The level of viral RNA in control groups 1 dpc was higher in kits than in adults, which is in line with the shorter average time to death observed in that group. Indeed, 1 dpc, the highest number of RNA copies (6.9 to 7.7 $\log_{10}$ copies/µL of serum) was obtained in kits which died just after sampling (32 to 39 h post-challenge), the intermediate number (2 to 3.8 $\log_{10}$ copies/µL) in adults for which death was observed later (between 48 to 54 h post-challenge), and the lowest level (9 to 16 copies/µL) corresponded to adults which succumbed after 70h. As for organs, the RNA load was higher in the serum of animal sampled just after death (up to 8.1 log 10 copies/µL of serum) than after ethical euthanasia (6.3 to 7.7 $\log_{10}$ copies/µL serum).

Viral RNA was not detected in the sera of vaccinated animals throughout the monitoring post-challenge phase.

Detection of viral RNA shedding

The results of the presence of viral RNA in the swabs of adult rabbits are given in Table 3. There was no RNA detectable 1 dpc in naso-conjunctival and rectal swabs, except for 2 control rabbits which were the first to succumb to the challenge. Two and 3 d post-inoculation, all the control rabbits were shedding viral RNA by both routes, except the surviving rabbit, which was only PCR-positive for nasal swab. Higher median copy number was found in naso-conjunctival swab (6.1 $\log_{10}$ copies *vs.* 4.2 $\log_{10}$ copies/rectal swab).

Between 2 and 3 dpc, the rate of positive naso-conjunctival swabs differed significantly between controls and vaccinated rabbits (8/8 in C10 group *vs.* 0/9 in V10 group, $F=17.0$, $P<0.0001$ for F test), as well as the rate of

Table 2: Detection of viral RNA in serum by RT-qPCR until 14 d post-challenge.

	Number of positive sera/total number of sera sampled			
Day Post-Challenge	1	2 to 3	8	14
C4	1/1* + 1/1[E]	1/1[D]	/	/
V4	0/2*	/	0/10*	0/10*
C10	5/5*	0/1*+3/3 [E]	0/1*	0/1*
V10	0/5*	/	0/9*	0/9*

* Animal without clinical signs; [D] animal sampled just after death; [E] animal sampled just after ethical euthanasia. C10, 10-wk-old control rabbits; C4, 4-wk-old control rabbits; V10, 10-wk-old vaccinated rabbits; V4, 4-wk-old vaccinated rabbits.

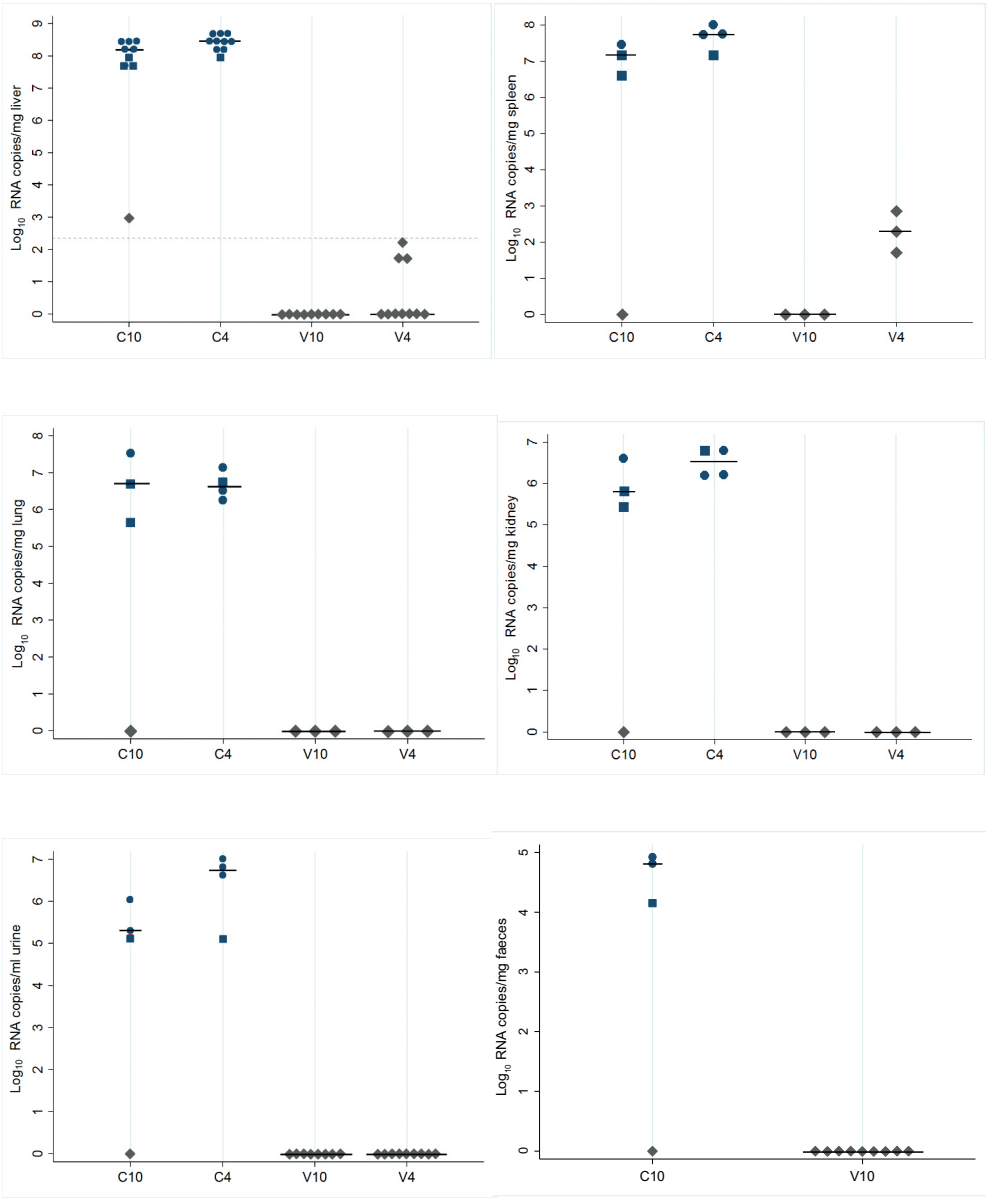


Figure 3: Viral RNA loads determined by specific RT-qPCR per mg of tissue (liver, spleen, kidney, lung) or mL of urine after GI.2-OLM.2017 challenge. C10, 10-wk-old control rabbits; C4, 4-wk-old control rabbits; V10, 10-wk-old vaccinated rabbits; V4, 4-wk-old vaccinated rabbits. Each dot represents individual data (blue circles and blue squares correspond to dead and ethically euthanised animals, respectively, whereas grey diamonds correspond to animal which survived to the challenge). The black line represents the median for each group (for C10 group, the surviving animal has not been taken into account). The dot line indicates the limit of detection of the RT-qPCR when applied to liver (2.3 $\log_{10}$ copies) which should be considered as the safe detection limit in that tissue (detection limit has not been studied for others tissues.

Table 3: Detection of viral RNA by RT-qPCR in naso-conjunctival and rectal swabs until 14 d post-challenge.

	Number of positive swabs/total number of swabs sampled			
Day Post-Challenge	1	2 to 3	8	14
Naso-conjunctival swabs				
C10	1/9*	4/4 [(D)] + 3/3[(E)] + 1/1*	0/1*	0/1*
V10	0/9*	0/9*	0/9*	0/9*
Rectal swabs				
C10	2/9*	4/4 [(D)] + 3/3[(E)] + 0/1*	0/1*	0/1*
V10	0/9*	0/9*	0/9*	0/9*

* Animal without clinical signs; [(D)] animal sampled just after death; [(E)] animal sampled just after ethical euthanasia. C10, 10-wk-old control rabbits; V10, 10-wk-old vaccinated rabbits.

positive rectal swabs (7/8 in C10 group *vs.* 0/9 in V10 group, F=13.4, P<0.0001). Until the end of the follow-up period (14 dpc), no viral shedding was detected via naso-conjunctival or faecal routes in vaccinated rabbits (V10).

In addition, 14 d post-challenge, none of the surviving rabbits (all the V4 and V10 groups and one C10) had viral RNA revealed by RT-qPCR in their urine, whereas a high load of viral RNA (5.1 to 7.1 $\log_{10}$ copies/mL) was excreted in urine of control rabbits which succumbed (Figure 3). The presence of viral genome in faeces, which was only investigated for adults, was not revealed by RT-qPCR in vaccinated rabbits at the end of the study, but was systematically found in each affected control after death (4.2 to 4.9 $\log_{10}$ copies/mg of faeces).

DISCUSSION

Eight years after the first identification of the new G.I.2/RHDV2/b genotype in France, and its rapid worldwide spread, the emerging lagovirus caused devastating effects on wild rabbit populations and represents a constant threat for rabbit farming. The virus is evolving rapidly and GI.2 strains with higher virulence were recently identified. Therefore, the aim of the present study was to assess the efficacy of Filavac K C+V vaccine against the GI.2-OLM.2017 challenge strain, representative of this most recent epidemiological evolution. This strain was isolated in France at the end of 2017 in a rabbit farm facing an RHD outbreak, with an unusually high mortality rate (80%) in unvaccinated kits. Its highly pathogenic nature was previously confirmed in SPF kits under experimental conditions (mortality rate of 100%). To our knowledge, only 2 small scale trials have been published on highly virulent GI.2 strains (Capucci *et al.,* 2017; Neimanis *et al.,* 2018). So, this challenge study was also the opportunity to describe the clinical course and virulence of the infection, in kits and adult rabbits, after experimental inoculation.

The first part of this discussion focuses on the description of the disease in control groups, as it provides the basis for the vaccine assessment. In this study, we could only observe acute RHD forms, as all the affected rabbits succumbed within 3 d (72 h) after inoculation. As recorded during preparatory tests, which included 3 experiments and a total of 30 kits between 4 to 5 wk of age (Le Minor, unpublished data), infection with GI.2-OLM.2017 was lethal to all kits. Likewise, the strain was also highly pathogenic to adult rabbits, with a case fatality rate (88.9%) similar to those induced by GI.1 viruses. For comparison with other challenge strains, GI.1-V4.1988 (reference strain V/RHD/4 isolated in 1988; Le Gall *et al.,* 1992) and GI.2-CH.2012 (collected in France in 2012), the full set of internal data was compiled between 2013 and 2018 (determination of the global mortality rate for each strain). This comparison is relevant, as all the experiments were carried out using the same dilution of inoculum and animals (same origin and genetic strain) as in the present study. A significant difference was found between mortality rates of GI.2 strains in adults (88.9% *vs.* 44% for GI.2-CH.2012, P=0.01 for Fisher's exact test) and in kits (100% *vs.* 52.4%, P=0.008 for Fisher's exact test), whereas no difference was found between GI.2-OLM.2017 strain and GI.1 strain (88.9% *vs.* 91.6% for GI.1-V4.1988, P=0.57 for Fisher's exact test). This significant increase in virulence is consistent with the recent experimental studies of Capucci *et al.* (2017) and Neimanis *et al.* (2018), who reported high case fatality rates (≥80%) after inoculation of Italian GI.2 strains and Australian GI.1bP-GI.2 strain, respectively. Both authors

hypothesised that GI.2 strains have evolved in their natural hosts since their emergence in 2010, and similar to what was proposed for GI.1 strains, selection pressure favours strains of higher pathogenicity.

In addition, taking into consideration exploratory trials (mentioned above), virulence data appear to be reproducible, unlike what has been described for the earliest GI.2 viruses. Indeed, Dalton *et al.* (2018) reported mortality rates in kits ranging between 10 to 50% within experiments using the same dose of RHDV-N11 isolate collected in Spain in 2011. Likewise, mortality rates induced by another Spanish GI.2 strain (2013) varied considerably among rabbit batches (from 0 to 75%), despite using rabbits from the single genetic strain and origin (Calvete *et al.,* 2018). In 2013, we also reported highly variable pathogenicity within experiments using the same dose of GI.2-CH.2012 challenge strain and similar conditions (age, origin, strain of the rabbits), with case fatality rates in the range of 30 to 67% (Le Minor *et al.,* 2013), which could be updated to 20-80% (Le Minor, unpublished data). Factors associated with this variability remained unclear, but individual innate immune system is possibly a key element (Capucci *et al.,* 2017; Calvete *et al.,* 2018). The decrease in this variability within individuals may suggest that host-virus interactions have changed.

As usually described in the case of (per) acute forms of RHD, most of the animals were found dead with no premonitory clinical signs. When observed, pyrexia and jaundice were the earliest symptoms, whereas haemorrhages (leading to "red eyes") were part of the terminal stage (a few hours before death). Lesions of similar morphology were found between kits and adult rabbits. Therefore, clinical evolution was similar between adult rabbits and kits, as GI.2-OLM.2017 strain induced comparable mortality rates, clinical signs and lesions. However, mean times to death (or ethical euthanasia) between age groups were significantly different and the course of the disease was, on av., 16 h quicker in kits. This explains the delay in the onset of clinical signs such as pyrexia in the adult control group.

Viral RNA was detected at high level in the serum, urine and organs of all control animals sampled at the final stage of the disease. Neimanis *et al.* (2018) reported similar ranges of RNA copies in liver, spleen, lungs, kidneys and sera of 5-wk-old kits dead after inoculation of a highly pathogenic recombinant strain. As expected, in our study, most of the samples collected just after death, which means after full disease progress, showed higher viral loads than those collected after human euthanasia. Therefore, due to this possible bias and to the small number of rabbits analysed, no statistical comparisons were performed between RNA loads of kits and adults.

Our results showed that early monitoring of RNA loads in serum could be a valuable indicator for clinical follow-up. Indeed, in affected controls, we observed a gradual increase in viral RNA loads as the infection progressed, until the final stage at which the highest levels were detected. The viral genome was detected as early as 31 h post-challenge in the serum of all the control animals sampled, with variable levels of RNA copies, which appeared to be indicative of residual time to death. Therefore, the highest RNA loads were found in kits which were already in the late stage of the disease, whereas the lowest loads corresponded to adults which survived until 2 and 3 dpc. It is interesting to note that Dalton *et al.* (2018) also reported higher levels of RNA in kit serum than in adult serum 1 d after inoculation of a mildly-pathogenic GI.2 strain (RHDV-N11 isolated in 2011).

To further our knowledge of the kinetics of viral RNA shedding, additional follow-up was carried out on adult rabbits via regular swabbing (naso-conjunctival and rectal) and faeces sampling (after death). Unlike viraemia, shedding of viral RNA was only detected in 2 animals 31 h post-challenge (for which death occurred few hours later). A peak of shedding was recorded between 2 and 3 d after challenge, as all control animals were shedding virus both by naso-conjunctival and faecal routes (associated with PCR-positive faeces), except the surviving rabbit, for which RNA was only detected in naso-conjunctival swab. After 3 dpc, all the swabs of this animal were PCR-negative. Dalton *et al.* (2018) made similar findings in rectal swabs of adult rabbits showing sub-clinical evolution after inoculation of middle-pathogenic GI.2, with a peak in RNA shedding 2 d post-challenge (12/12) and a decrease 7 dpc (2/6). In contrast to our results, this was not associated with nasal shedding.

Vaccination did not induce local or general adverse reactions in 4 and 10-wk-old SPF rabbits. Seroconversion against GI.1 virus validates vaccine uptake within 15 d after administration.

To our knowledge, this is the first report of a vaccine efficacy study against a highly pathogenic GI.2 challenge.

Our results show that, 7 d after immunisation with inactivated Filavac K VHD C+V vaccine, all the vaccinated animals were fully protected against highly pathogenic GI.2-OLM.2017 strain. The vaccination prevented mortality, general

signs and gross lesions in kits and adult rabbits, and statistically significant differences were observed between survival rates of vaccinated and respective control groups. Vaccinated kits presented a regular development with normal average daily gain (AGD), and, unlike control group, did not show any significant increase in mean rectal temperature for the 14 d of follow-up after challenge. In addition, vaccination significantly protected from the detection of viral RNA in serum. In adult groups, we also demonstrated that vaccine significantly prevented detectable RNA shedding via naso-conjunctival and rectal routes. The non-detection of viral shedding via a highly sensitive molecular method during the acute phase of the disease, and until the end of the study, is strong evidence in favour of the absence of asymptomatic spreading among vaccinated rabbits. This is a key element for management of the disease in infected farms.

At the end of the challenge, we investigated by RT-qPCR the persistence of viral RNA in different organs and excreta, as previous studies demonstrated that viral RNA can be detected in convalescent rabbit tissues, urine and faeces months after infection, with a high accumulation in liver and spleen, which are the major target organs of the virus (Shien *et al.,* 2000; Gall *et al.,* 2007; Calvete *et al.,* 2018). Two weeks after challenge, viral RNA copies were not detected by RT-qPCR in the liver, rate, lungs, kidneys, faeces and urine of vaccinated adult rabbits. Similar findings were made for kits, except that very low levels of viral genomes were present in the liver of 3 (out 10) and in the spleen of 3 (out 3) rabbits sampled (faeces were not analysed in that age group). Is it important to note that, in the liver, the numbers of viral RNA copies were below the safe detection limit of the PCR method (2.3 $\log_{10}$ copies/µg), and the results should therefore have been reported as negative for the 3 kits. In addition, these results are consistent with the detection of residual inactivated vaccine strain, as we know that 30% of vaccinated animals still show a low number of copies (<2.3 $\log_{10}$ copies/µg) 3 wk after vaccination. As already discussed by Carvalho *et al.* (2017), due to high sensitivity of the RT-qPCR method, inactivated vaccine-derived RNA could indeed originate weakly positive RT-qPCR results, if still present in the tissues. Unintentional intravasal injection, enhanced blood permeability at the site of injection, systemic distribution of viral RNA via phagocytic cells or the association of inactivated virus with erythrocytes are some of the hypotheses.

Unfortunately, due to the high pathogenicity of the GI.2-OLM.2017 strain, comparative data are missing, as no kit survived in the control group. It may however be noted that a 5 to 12-times higher level of viral RNA (above the safe detection limit) was detected in the liver of the only surviving control (adult rabbit). The level of RNA persistence in this control (2.9 $\log_{10}$ copies/µg liver) is consistent with previously internal data collected for GI.2-CH.2012 challenge, after which 100% of surviving controls were still PCR-positive 14 dpc (range: 3 to 4.5 $\log_{10}$ copies) (Le Minor, unpublished data). In addition, several experimental studies showed that vaccination against GI.1 or GI.2 lagoviruses, regardless of the type of vaccine (inactivated vaccines, recombinant or an experimental subunit), is unable to ensure complete "sterile immunity" after challenge, as carriers of low amounts of viral RNA are identified (Gall and Schirrmeier, 2006; Spikey, *et al.,* 2012; Read and Kirkland, 2017). However, until now, none of the experiments have been able to prove that the persistence of RNA in different organs, including spleen and liver, is linked to the persistence of infectious virus particles (Shien *et al.,* 2000; Forrester *et al.,* 2003; Gall *et al.,* 2007; Strive *et al.,* 2010).

Although very satisfying results were obtained in kits, results have to be confirmed in the presence of maternal antibodies, and the duration of immunity against challenge needs to be assessed in this age group.

In conclusion, GI.2-OLM.2017 strain induced acute RHD with high mortality rates, similar to those caused by classic GI.1 viruses, both in adult rabbits and kits. Immunisation with Filavac K V+C vaccine, at 4 and 10 wk of age, still provides a full and early protection against mortality and clinical signs after experimental challenge with this highly pathogenic GI.2 strain. Therefore, we demonstrated that, despite the rapid evolution of GI.2 strains since 2010, the protection conferred by the vaccine remains adequate. In the current epidemiological context, with the coexistence of both GI.1 and GI.2 genotypes in some countries and the identification of multiple recombinant viruses, it seems crucial to keep a broad spectrum vaccination with the association of strains from both genotypes.

Acknowledgements: The authors would like to thank Delphine Artusse, Beatrice Arrignon, Martine Brandeau, Jérôme Martineau and Johan Faucoulanche for their helpful laboratory work.

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