Vaccine 37 (2019) 4195–4203

Contents lists available at ScienceDirect

Vaccine

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

Characterization of protective humoral and cellular immune responses against RHDV2 induced by a new vaccine based on recombinant baculovirus

Claudia Müller [a], Reiner Ulrich [a], Jan Schinköthe [a], Marcus Müller [a,b], Bernd Köllner [a,*]

[a] Friedrich-Loeffler-Institute, Institute of Molecular Virology and Cell Biology, Department of Experimental Animal Facilities and Biorisk Management, Institute of Immunology, Greifswald-Insel Riems, Germany
[b] IDT Biologika Riems, Greifswald-Insel Riems, Germany

ARTICLE INFO

Article history:
Received 8 August 2018
Received in revised form 4 April 2019
Accepted 19 April 2019
Available online 8 June 2019

Keywords:
RHDV
Recombinant vaccine
Protection
Immune response

ABSTRACT

Rabbit hemorrhagic disease (RHD) is a lethal disease in rabbits caused by RHD virus (RHDV). Protection is only possible through vaccination. A new virus variant (RHDV2) which emerged in 2010 in France differed from the classical RHDV1 variant in certain aspects and vaccines against RHDV1 induced limited cross protection only. In a previous study, we designed a recombinant baculovirus based RHDV2-VP1 vaccine, which provided a protective immunity in rabbits against RHDV2. In the present study this newly created vaccine is characterized with regard to onset and duration of protection, and possible cross protection against classical RHDV1. Furthermore, humoral and cellular immune mechanisms in vaccinated and infected rabbits were analyzed. In all experiments, the recombinant vaccine was compared to a conventional liver-based RHDV2 vaccine.

The RHDV2-VP1 vaccine induced a protective immune response already seven days after single vaccination and fully protected for at least 14 months. A booster vaccination 21 days after the first had a negative influence on long-term protection. The cross protection provided by the RHDV2-VP1 vaccine against classical RHDV1 was limited since only 50% of vaccinated rabbits survived the infection. Conclusively, the new, baculovirus-based RHDV2-VP1 vaccine has the potential to protect rabbits against the infection with RHDV2, blocks completely the disease progression and prevents the spread of RHDV2 at the population level.

1. Introduction

Rabbit hemorrhagic disease virus (RHDV), first discovered in 1984 in China in angora rabbits imported from Germany [38], is the causative agent of rabbit hemorrhagic disease (RHD). RHDV belongs to the family Calicivirus, genus Lagovirus.

The infection proceeds to fatal disease in rabbits, and induces significant losses in the rabbit fur and meat industry [26]. After the first outbreak the virus spread rapidly through Europe and afterwards Africa, the Americas, Asia, Europe and Oceania [1].

The disease progression is in most cases acute, with severe necrotizing hepatitis and necrotic lesions variably be found in other organs (lungs, kidneys and spleen). Rabbits are fully susceptible from the age of 9 weeks, and the mortality rate is 70–100% [1]. The peracute disease course leads to death within 1–2 days post infection without any clinical symptoms. In prolonged forms infected rabbits display lethargy, anorexia and death after 1–2 weeks [1].

In 2010 a new virus variant, RHDV2, was discovered in France which is currently spreading through Europe and has already reached Australia [19,3,37,6,7,16]. RHDV2 causes the same symptoms and pathological alterations as the 'classical' RHDV1 although a prolonged course of the disease and lower mortality rates were described [19]. RHDV2 has the ability to infect young 4 weeks old rabbits [19] as well as certain RHDV1-resistant hare species [30,5].

Because RHDV1 and RHDV2 do not replicate in cell cultures anti-RHDV1 vaccines are mainly based on liver preparations of infected rabbits. Also new vaccines against RHDV2 are produced from homogenates of infected liver (Eravac, Laboratorios Hipra S.A., Spain; Filavac VHD K C + V, Filavie, France). Such vaccines from livers of heavily sick rabbits are critical regarding animal welfare issues.

* Corresponding author at: Friedrich-Loeffler-Institute, Institute of Immunology, Greifswald-Insel Riems, Germany.
E-mail address: bernd.koellner@fli.de (B. Köllner).

https://doi.org/10.1016/j.vaccine.2019.04.061
0264-410X/

Therefore, aims of this study were to develop a recombinant anti-RHDV2 vaccine using the baculovirus expression system which induce a long lasting protective immunity against RHDV2, to analyze onset, duration and cross protection induced by this recombinant RHDV2 vaccine and to characterize anti-RHDV2 specific humoral and cellular immune response of rabbits after vaccination.

2. Methods

2.1. Cells

SF9 (*Spodoptera frugiperda*) cells were cultivated in Grace's supplemented insect cell medium with 10% fetal calf serum (FCS), 100 U of penicillin per ml, and 100 μg of streptomycin per ml at 27 °C and 2.5% CO_2.

2.2. Preparation of vaccine candidates

In all experiments a recombinant baculovirus expressing VP1 of RHDV2 was used as vaccine [24]. Briefly, an artificial open reading frame (ORF) coding for both the VP1 protein of RHDV2 (Genbank Acc-NoFR819781; [19] and the green fluorescent protein (GFP) for infection control was constructed using the Bac-to-Bac® Baculovirus Expression System (Thermo Fisher Scientific, Germany) to generate recombinant baculoviruses. SF9 cells were infected with the recombinant baculoviruses at MOI 1 and incubated at 27 °C and 2.7% CO_2 for 3 days. For harvesting of RHDV-VP1 virus like particles (VLPs, see Suppl. Fig. 1), the cells were detached and centrifuged with 1500 rpm at 4 °C for 20 min. The recombinant vaccine (referred to as "recRHDV2-vacc") was prepared with a freezing-thawing cycle of the cell pellet, resuspension in PBS and ultrasound disintegration of cells. The antigen content in the recRHDV2-VLPs vaccine stocks was determined using Hemagglutination assay and indirect ELISA Kit (Ingenasa) as described below.

The "recRHDV2-vacc" was used in comparison to a conventionally prepared liver vaccine inactivated with binary ethylenimine from RHDV2 (strain "Werne") infected rabbits (referred to as "convRHDV2-vacc"; kindly provided by Dr. Schirrmeier, FLI Riems).

Both vaccine stocks were adjusted to the desired hemagglutination units in PBS and mixed with aluminum hydroxide following the standard operation procedure for the RHDV1 vaccine "Cunivak RHD" (IDT Biologika, Insel Riems, Germany). A negative control vaccine was produced in SF9 cells infected with recombinant baculoviruses at MOI 1 expressing GFP but no VP1 (referred to as "recbacGFP-vacc") similar to the "recRHDV2-vacc".

2.3. Animal experiments

All animal trials were performed following the acquirements of the EU directive 2010/63; the EG recommendation 2007/526/ and confirmed by German animal welfare committee (No. LALLF-7221.3-1-025/15).

2.3.1. Animals

For all trials 10–20 weeks old ZiKa-hybrid rabbits of both sexes from a commercial rabbit farm were used. All animals were clinically examined and the absence of antibodies against RHDV2 was verified by ELISA. The rabbits were fed with commercial rabbit food (ssniff Spezialdiäten GmbH, Germany) and water *ad libitum*.

2.3.2. Immunization

Rabbits were vaccinated into the *musculus quadriceps femoris* either with 0.5 ml "recRHDV2-vacc", "convRHDV2-vacc" or "recbacGFP-vacc". Respective hemagglutination unit (HU) contents for every trial are specified in the results section. Non-vaccinated rabbits served as negative control in each trial.

2.3.3. Challenge infection

The challenge infection in all trials was done by injection into *musculus quadriceps femoris* with 2560 hemagglutination units (HU) of challenge virus RHDV2 strain "Werne" or RHDV1 strain "Eisenhüttenstadt". After challenge, the health status of all rabbits was monitored repeatedly per day and rectal body temperature was measured twice a day over two weeks.

Blood was sampled as described below until the rabbits were euthanized in a moribund stage or died. 14 days after challenge all remaining animals were euthanized in accordance with animal welfare regulations and blood and organ samples were taken and prepared for further analysis.

2.3.4. Design of vaccination-challenge trials

Determination of minimal effective dose. Three groups of four rabbits were vaccinated with a single dose of 256 HU or 512 HU or 1024 HU of "recRHDV2-vacc", respectively. Four rabbits were vaccinated with 512 HU of the "convRHDV2-vacc". Four non-vaccinated rabbits served as control group. Blood serum samples were taken weekly over four weeks as described. All rabbits were challenged 14 days post vaccination. Macroscopic and histopathologic examination and organ sampling was done after challenge as described.

Onset of vaccine induced anti-RHDV2 immune response. Four rabbits were vaccinated either with 1024 HU of the "recRHDV2-vacc" or 512 HU of the "convRHDV2-vacc". Four non-vaccinated rabbits served as control group. All rabbits were already challenged seven days post vaccination as described. All rabbits which died during the course of the experiment underwent necropsy and histopathologic examination. From surviving vaccinated rabbits blood was sampled weekly for four weeks and then monthly over 14 months after vaccination for long term observation.

Duration of vaccine induced anti-RHDV2 immune response. 10 rabbits were single vaccinated with 1024 HU of the "recRHDV2-vacc". Further 8 rabbits received a booster vaccination 21 days later with 1024 HU of the "recRHDV2-vacc". Eight rabbits vaccinated with 512 HU of the "conv-RHDV2-vacc" served as positive control and eight non-vaccinated rabbits as negative control. From all rabbits blood was sampled weekly over four weeks and then monthly. Four rabbits of each group were challenged six months post vaccination; the remaining rabbits were challenged 14 months after vaccination with RHDV2 as described. Blood serum samples were collected weekly after challenge. All rabbits which survived were euthanized and underwent necropsy and histopathologic examination two weeks after the challenge infection.

Cross protection provided by vaccine-induced anti-RHDV immune response. Two groups of four rabbits each were vaccinated with 1024 HU "recRHDV2-vacc" or 512 HU of "conv-RHDV2-vacc", respectively. Two groups of four non-vaccinated rabbits served as control groups. After vaccination blood serum samples were taken weekly over four weeks. Each vaccinated group and control group was challenged 14 days post vaccination with either homologous RHDV2 or heterologous RHDV1. All rabbits which survived were euthanized and underwent necropsy and histopathologic examination two weeks after the challenge infection.

Evaluation of immune response after vaccination with baculoviruses expressing GFP. As a further control nine rabbits were vaccinated with recombinant Baculovirus expressing GFP only "recbacGFP-

vacc". At day 14 post vaccination this group was challenged with RHDV2. Blood samples were taken each 12 h after challenge.

2.3.5. Blood sampling

1 ml blood was sampled from ear veins into EDTA pretreated tubes (Sarstedt, Germany) for leukocyte isolation and 200 μl blood was sampled for serum collection in intervals described above.

2.3.6. Necropsy

All rabbits underwent necropsy and organ samples from heart, lung, spleen, liver, kidney, intestine and brain were fixed in 10% formalin, embedded in paraffin wax, sectioned at 2–4 μm thickness, mounted on glass slides, stained with hematoxylin and eosin and assessed for histopathological changes by light microscopy.

2.4. Evaluation of RHDV viral load in liver

The viral load in rabbit liver tissue after RHDV2 infection was determined using about 200 mg liver samples homogenized after adding of Hank's balanced salt medium using Qiagen Tissue Lyser II for 2 min at 30 Hz. The lysed tissue was centrifuged at 14,000 rpm at 4 °C for 5 min with an Eppendorf centrifuge and supernatant was taken for further analysis.

2.5. Hemagglutination assay

The hemagglutination assay (HA) was performed as titration (2-fold) according to OIE standard procedure using 50 μl of each liver sample and 1% human erythrocytes of blood group 0. A liver homogenate with predetermined RHDV2 (strain "Werne") titer was used as positive control, isotonic phosphate buffer as negative control. The HA titer was expressed as the value of the highest dilution resulting in complete hemagglutination.

2.6. Antigen ELISA

100 μl of each liver sample were tested in duplicates using the commercial ELISA Kit "Ingezim RHDV DAS R.17.RHD.K2" by Ingenasa according to manufacturer's protocol. A liver sample from a rabbit infected with RHDV2 strain "Werne" served as external positive control. Absorbance was measured at 450 nm with an ELISA reader (Spectra, Tecan).

2.7. Quantitative real time RT-PCR

RNA was purified from the liver samples using the QIAamp Viral RNA Mini Kit (Qiagen, Germany) according to manufacturer's protocol. For each sample 5 μl of an internal process control RNA ("IC-RNA", [17]; supplement Table 1) was added.

The amount of RHDV-RNA in liver samples, was analyzed using 5 μl of purified RNA by qRT-PCR method ([13]; supplement table 1) using the SuperScript™ III One-Step RT-PCR (Invitrogen, Germany). RNase free water served as no template control ("NTC"), purified RHDV2-RNA with a threshold cycle value (ct) of 33 or standard RHDV1-RNA with 2×10^6 copies/μl served as positive control ("PC"). The PCR was run for 30 min at 50 °C followed by 2 min at 94 °C and 42 cycles of 30 s at 94 °C, 45 s at 55 °C and 45 s at 68 °C (MX3005P; BioRad Germany) and analyzed with the software program "MxPro". The amount of viral RNA was calculated using the ΔΔ2ct method [20] and expressed as 2 fold titers of viral RNA.

2.8. Measurement of RHDV specific serum antibodies

The titer of RHDV1 or RHDV2 specific antibodies in serum samples were analyzed in an indirect ELISA. Briefly, the rabbit sera were incubated at 37 °C for 1 h in two-fold dilutions in PBS/0.05% Tween20/5% horse serum onto 96-well ELISA plates (Microlon® 200 96 W Microplate, Greiner, Germany) coated with 100 μl/ well of purified RHDV1 or RHDV2 antigen, respectively in 0.02 M Tris, 0.15 M NaCl. After 3 times washing with PBS/T 100 μl anti-rabbit IgG POD conjugate per well (Dianova, Germany) in PBS/T were incubated at 37 °C for 1 h. After three more washing steps 100 μl/ well OPD substrate solution (Sigma-Aldrich, Germany) was incubated for 30 min at RT in the dark. The reaction was stopped with 50 μl 4 M H_2SO_4 per well and absorbance was measured at 492 nm (Spectra, Tecan, Germany).

2.9. Flow cytometric analysis of cellular immune response

PBL were prepared by density gradient centrifugation from 1 ml of EDTA blood diluted 1:4 v/v with PBS, 0.01% 1 mM EDTA (PBS/E) on 3 ml of Pancoll (1077 g/ml; Pan-Biotech, Germany) for 30 min at 1800 rpm in an Eppendorf centrifuge. The cells at the interface were collected, washed with PBS/E and 2×10^5 cells/ well in PBS/E were incubated with combinations of different monoclonal antibodies specific for leukocyte differentiation markers at 4 °C for 30 min (supplement table 2). After washing with PBS/E the labelled cells were incubated with isotype specific fluorochrome antibody conjugates (supplement table 2) for further 30 min at 4 °C. After final washing the cells were resuspended in 300 μl PBS/E and analyzed in FACScalibur (Becton Dickinson, Germany).

3. Results

3.1. Non-vaccinated or rabbits vaccinated with "recbacGFP-vacc" displayed only very limited natural resistance

Almost all non-vaccinated rabbits developed poor general condition, reduced food intake, high fever over 40 °C, and finally died or were euthanized for humane reasons (Table 1).

Necropsy and histopathology of these rabbits confirmed the RHD typical necrotizing hepatitis (see also Table 1 + Supp. Tab. 3). In *post mortem* liver samples of these rabbits an up to 220 times higher high viral load was detected between 36 and 96 h post challenge than in the three survivors (Fig. 1).

All three surviving non-vaccinated animals displayed mild clinical symptoms and two developed fever over 40 °C for four days. The lymphohistiocytic periportal hepatitis with variable amounts of heterophils and multinucleated giant cells detected in two of these three rabbits 2 weeks after the challenge indicated a resorptive sequelae to a previous hepatocellular damage (Suppl. Fig. 2). RHDV-RNA was still detectable in liver samples (Table 1).

The infection with RHDV2 induced a strong depletion of $CD4^+$ as well as $CD8^+$ T-cells shortly after infection in non-vaccinated rabbits with nearly no detectable $CD8^+$ T-cells in blood (Fig. 2). In contrast, increased $CD4^+$ and $CD8^+$ T-cells as well as increased a-RHDV2 antibody titers were observed after challenge in the survivors.

Also, all nine animals vaccinated with the empty control "recbacGFP-vacc" died between 30 and 125 h after challenge with poor general condition before death and high fever over 40 °C (Table 1). Necropsy and histopathology confirmed the same RHD typical necrotizing hepatitis (Suppl. Fig. 2). In *post mortem* liver samples a high viral load was detected (Table 1).

3.2. An immunization with RHDV2 vaccine formulation provided complete protection against RHDV2 induced disease

36 out of 37 rabbits vaccinated with the "recRHDV2-vacc" survived after RHDV2 challenge infection from which 33 rabbits did not show any RHD-specific clinical symptoms (Table 1). However,

Table 1
Summarized overview about clinical outcome, pathological changes and viral load in non-vaccinated rabbits or rabbits vaccinated with the newly established "recRHDV2-vacc" in comparison to "convRHDV2-vacc" and "recbacGFP-vacc" after challenge with RHDV2.

Vaccination	recRHDV2-vacc		convRHDV2-vacc	Non-vaccinated		recbacGFP-vacc
Challenge with	RHDV2	RHDV2	RHDV2	RHDV2		
Clinical outcome						
No. of animals	37		19	20		9
Survived	36		19	3		0
Died		1	0		17	9
Mean survival time, h	336	34	336	336	42	48
Clinical symptoms/pathological findings						
Fever >40 °C	4	0	2	2	9	7
Necrotizing hepatitis	0	1	0	0	17	9
Lung edema	0	1	0	0	17	9
Hemorrhages	0	1	0	0	17	9
Viral load						
RNA, q-RT-PCR; 2^e	0.17	27.84	0	5.14	31.15	31.5
viral particle, HA, 2^e	0	12	0	0.3	11.8	11.33
VP60, ELISA, OD	0.05	0.72	0.05	0.04	1.03	0.98

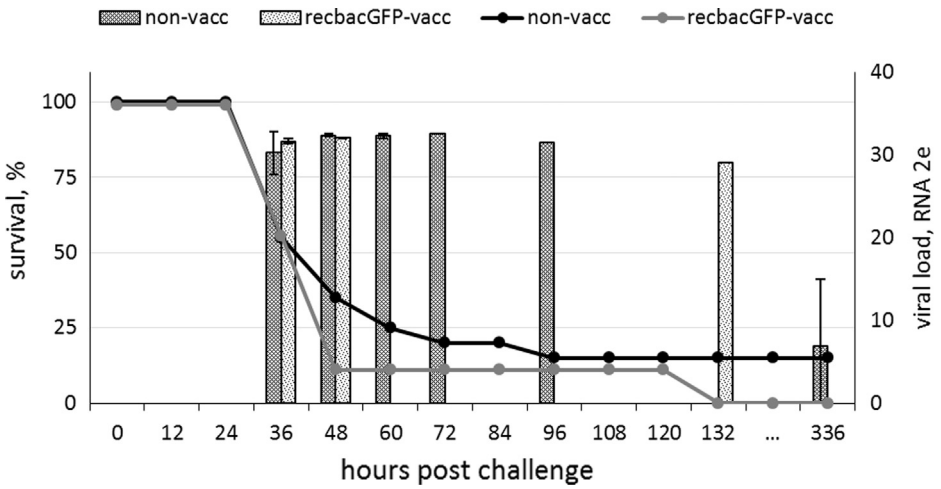

Fig. 1. Cumulative mortality (lines) of differentially treated rabbits after infection with RHDV2 and corresponding viral load (columns) in liver samples taken from these rabbits. Rabbits were vaccinated with a "recbacGFP-vacc" and compared to non-vaccinated rabbits. Note the high viral load in rabbits that died after infection before end of trial in comparison to survivors at 336 h post challenge.

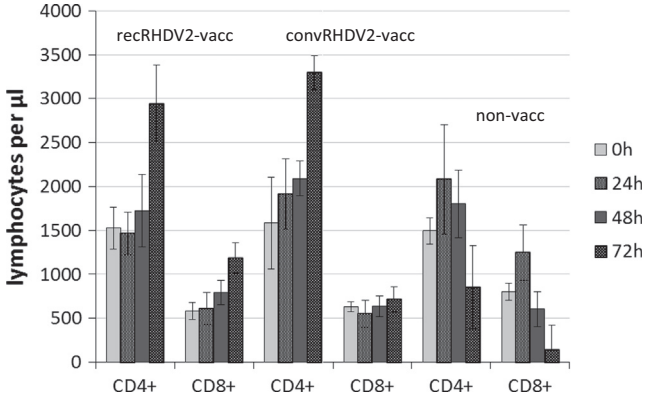

Fig. 2. Kinetics of CD4+ and CD8+ T-cells in blood of rabbits vaccinated with "recRHDV2-vacc" or "convRHDV2-vacc" compared to non-vaccinated rabbits after infection with RHDV2. Within 36 h the total number of T-cells in blood drops down to about 50% (CD4+ T-cells) or about 10% (CD8+ T-cells) in non-vaccinated rabbits compared to normal value. Note the increase of CD4+ T-cells in blood after vaccination with both vaccines in contrast to the increase of CD8+ T-cells after vaccination with the recombinant vaccine only.

half of the rabbits displayed a lymphohistiocytic periportal hepatitis with rare multinucleated giant cells and in 4 of 31 rabbits a splenic hyperplasia two weeks after the challenge was detected (Suppl. Tab. 3). Four animals developed fever over 40 °C after challenge at one single time point. All rabbits vaccinated with the control "convRHDV2-vacc" survived RHDV2 challenge without RHD specific clinical symptoms (Table 1). Two animals developed fever over 40 °C after challenge infection at one single time point. Macroscopic changes were observed in a few rabbits similar to the "recRHDV2-vacc" immunized animals (Table 1 + Suppl. Tab. 3). In liver samples of the surviving vaccinated animals no RHDV-RNA or -antigen was detected. In contrast, in the one vaccinated rabbit which died a high amount of both RHDV2-RNA and -antigen was found (Tables 1 + 4b).

The induction of a protective humoral immunity after vaccination was combined with a stimulation of both $CD4^+$ and $CD8^+$ blood T-cells but with a different pattern: the "recRHDV2-vacc" induced an increase of both blood T-cell populations; the "convRHDV2-vacc" induced an increase of $CD4^+$ T-cells only (Fig. 2).

3.3. A low dose of rec-RHDV2-vacc induced protection and a protective anti-RHDV2 antibody titer.

A protective immune response could be induced already with a low dose of "recRHDV2-vacc" of 256 HU after a single immunization without any clinical signs of RHD and pathological findings

characteristic for RHD. No viral load was detected in liver samples of vaccinated rabbits at the end of the trial (Table 2). Also all vaccinated rabbits immunized with "convRHDV2-vacc" survived the RHDV2 challenge. Vaccination using "recRHDV2-vacc" induced low, but protective titers of RHDV2 specific antibodies at day 14 post vaccination which did not correlate to the vaccine dose. These titers increased strongly after challenge with RHDV2 in all vaccinated rabbits (Table 2). Due to the findings for all other trials a dose of 1024 HU of "recRHDV2-vacc" was used.

3.4. The protective immune response against RHDV2 infection was induced already seven days post vaccination

Rabbits immunized once either with "recRHDV2-vacc" or "convRHDV2-vacc" survived challenge with RHDV2 with no clinical signs of RHD disease already seven days post vaccination. However this protection was not correlated in all rabbits with high anti-RHDV2 antibody titers in sera sampled before challenge. After challenge the titers of RHDV2 specific antibodies increased remarkably (Table 3a).

3.5. A single immunization with the "recRHDV2-vacc" induces a long lasting immunity

Rabbits immunized once with the "recRHDV2-vacc" developed a long lasting immunity which was comparable to the induced protection after a single vaccination with "convRHDV2-vacc" (Table 4a). All vaccinated rabbits survived a RHDV2 infection six months after vaccination without any clinical signs or pathological findings characteristic for RHD. Moreover, no viral load was detected in livers of vaccinated rabbits at the end of the trial (Table 4a). Fourteen months after vaccination all rabbits immunized once with " recRHDV2-vacc" or "convRHDV2-vacc" survived the RHDV2 infection with no clinical signs.

However, all rabbits immunized a second time 21 days after the first vaccination developed clinical signs like apathy and low food uptake. One of them died 34 h after challenge infection with RHDV2 with a high viral load in the liver (Tables 1 + 4b) and RHD typical necrotizing hepatitis was confirmed (see also Table 1). In the three surviving "recRHDV2-vacc" boostered rabbits a lymphohistiocytic periportal hepatitis but no replication of RHDV2 in liver was observed (Table 4b).

The kinetics of RHDV2 specific antibody titers increase steadily after vaccination with both "recRHDV2-vacc" and "convRHDV2-vacc" and declined slowly till six months. One rabbit that received a prime-boost vaccination did not show antibody titers after six months (data not shown) but survived RHDV challenge without signs of RHD.

In sera of twice "recRHDV2-vacc" immunized rabbits a stronger decline of RHDV2 specific antibody titers was measured in comparison to once "recRHDV2-vacc" or "convRHDV2-vacc" immunized rabbits (Table 4a + b; Fig. 3). The "recRHDV2-vacc" vaccinated rabbit which died after challenge infection after 14 months developed no RHDV2 specific serum antibodies (data not shown).

In comparison, rabbits one time immunized with "recRHDV2-vacc" or "convRHDV2-vacc" and challenged with RHDV2 seven days after vaccination (see Section 3.4) completely survived second RHDV2 infection 14 months without RHD typical clinical symptoms, pathological alterations in inner organs or viral load in livers. Rabbits of those two vaccine groups developed high antibody titers which did not decline till the second challenge infection (Table 3b).

Table 2
Clinical outcome, viral load in livers and anti-RHDV2 antibody titers in sera of rabbits vaccinated with different doses of "recRHDV2-vacc" in comparison to rabbits vaccinated with "convRHDV2-vacc" and non-vaccinated rabbits after challenge with RHDV2.

Group	surv/died	mst, h	RNA, q-RT-PCR; 2^e	Viral particle, HA, 2^e	VP60, ELISA, OD	Antibody titers		
						d0	d14	d28
recRHDV2-vacc 1024	4/0	336	0	0	0.06	1:50	1:263	1:20,000
recRHDV2-vacc 512	4/0	336	0	0	0.06	1:50	1:88	1:2400
recRHDV2-vacc 256	4/0	336	0	0	0.06	1:50	1:338	1:10,400
convRHDV2-vacc	4/0	336	0	0	0.07	1:50	1:1400	1:25,600
non-vacc	1/3	336/41	18.32/32.18	1/12.67	0.08/1.01	1:50	1:50	1:25,600

d 0 = day of vaccination; d 14 = day of challenge; d 28 = end of trial; mst = mean survival time.

Table 3
Clinical outcome, viral load in livers and long-term observation of anti-RHDV2 antibody titers over 14 months in rabbits single vaccinated with "recRHDV2-vacc" (A) after first challenge infection seven days post vaccination, and (B) after second challenge infection 14 months post vaccination. Rabbits vaccinated with "convRHDV2-vacc" and non-vaccinated rabbits served as control.

(A) Challenge after 7 days

Group	surv/died	mst, h	RNA, q-RT-PCR; 2^e	Viral particle, HA, 2^e	VP60, ELISA, OD	Antibody titers		
						d0	d7	d28
recRHDV2-vacc	4/0	336	n.d.	n.d.	n.d.	1:25	1:113	1:20800
convRHDV2-vacc	4/0	336	n.d.	n.d.	n.d.	1:63	1:150	1:25600
non-vacc	1/3	336/51	n.d./32.5	n.d./11	n.d./1.12	1:50	1:88	1:25600

(B) Challenge after 14 months

Group	surv/died	mst, h	RNA, q-RT-PCR; 2^e	Viral particle, HA, 2^e	VP60, ELISA, OD	Antibody titers				
						d28	d149	d427	d434	d441
recRHDV2-vacc	4/0	336	0	0	0.05	1:20800	1:11200	1:3900	1:19200	1:16000
convRHDV2-vacc	4/0	336	0	0	0.05	1:25600	1:14400	1:3600	1:25600	1:16000
non-vacc survivor	1/0	336	0	0	0.05	1:25600	1:25600	1:12800	1:25600	1:12800
non-vacc	0/4	34	27.3	12	0.74					

n.d. = not determined after the first challenge; d 0 = vaccination; d 7 = day of first challenge; d 28 = 21 days after challenge infection with RHDV2; d 427 = second challenge infection with RHDV2 after 14 months observation; d 441 = end of trial; mst = mean survival time.

Table 4
Clinical outcome, viral load in livers and long-term observation of anti-RHDV2 antibody titers in rabbits vaccinated once or twice with "recRHDV2-vacc" (A) over six months post vaccination and (B) over 14 months post vaccination. Rabbits vaccinated with "convRHDV2-vacc" once and non-vaccinated rabbits served as control. Note: Different group sizes due to losses of rabbits during the year.

(A) challenge after 6 months

Group	surv/died	mst, h	RNA, q-RT-PCR; 2^e	Viral particle, HA, 2^e	VP60, ELISA, OD	Antibody titers						
						d0	d21	d58	d156	d203	d210	d217
recRHDV2-vacc 1x	4/0	336	0	0	0.05	1:63	1:1113	1:700	1:1900	1:1450	1:19200	1:25600
recRHDV2-vacc 2x	4/0	336	0.5	0	0.05	1:50	1:1800	1:6400	1:800	1:663	1:25600	1:25600
convRHDV2-vacc 1x	4/0	336	0	0	0.05	1:50	1:8800	1:10800	1:14000	1:4400	1:25600	1:25600
non-vacc	1/3	336/32	2.2/32.8	0/13	0.05/0.96							

(B) challenge after 14 months

Group	surv/died	mst, h	RNA, q-RT-PCR; 2^e	Viral particle, HA, 2^e	VP60, ELISA, OD	Antibody titers						
						d0	d21	d58	d156	d427	d434	d441
recRHDV2-vacc 1x	5/0	336	0.8	0.4	0.05	1:60	1:2000	1:3280	1:1760	1:1040	1:17920	1:25600
recRHDV2-vacc 2x	3/1	336/34	0/27.8	0.3/12	0.05/0.72	1:50	1:1213	1:2900	1:700	1:113	1:9067	1:10667
convRHDV2-vacc 1x	3/0	336	0	0.3	0.04	1:50	1:5333	1:10667	1:12800	1:2667	1:25600	1:17067
non-vacc	0/4	34	27.3	12	0.74							

d 0 + d 21 = vaccination; d 203 = challenge after 6 months observation; d 217 = end of trial; d 427 = challenge after 14 months observation; d 441 = end of trial; mst = mean survival time.

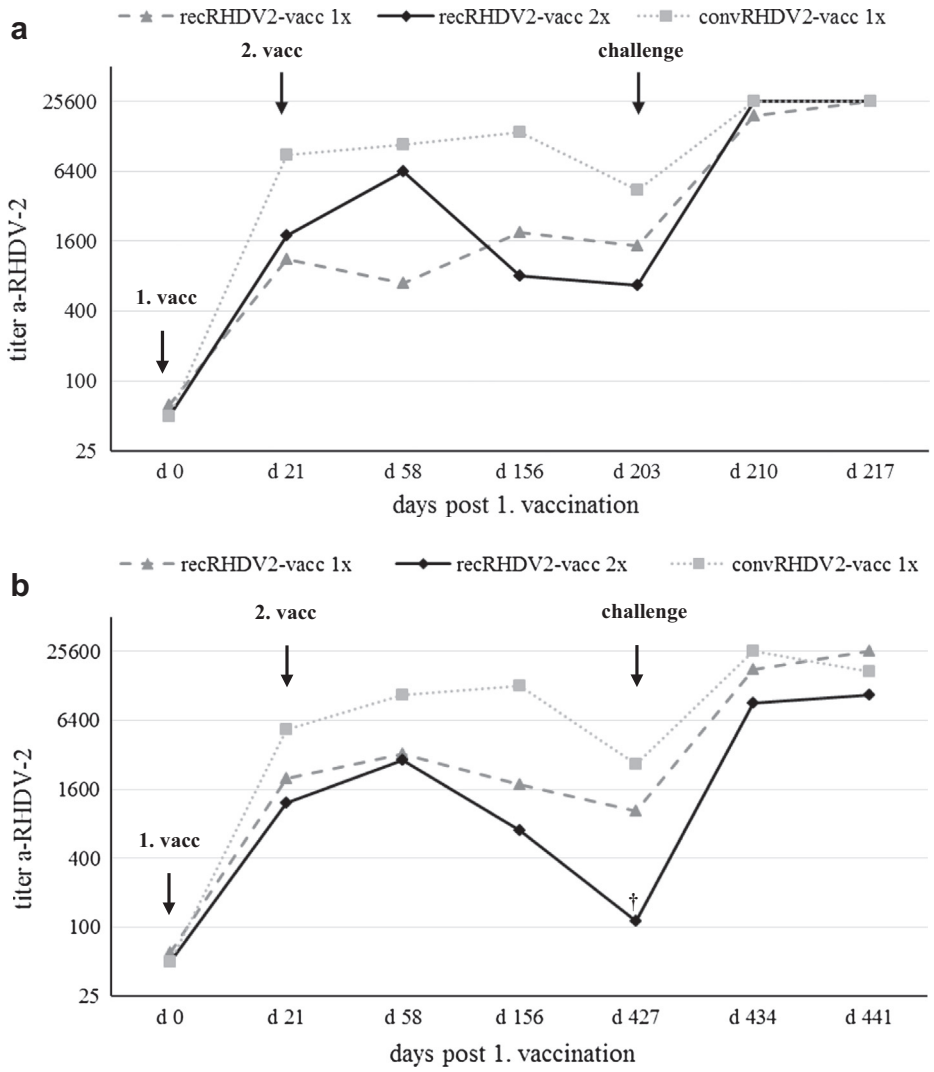

Fig. 3. Long-term observation of anti-RHDV2 antibody titers in sera of rabbits after vaccination once or twice with "recRHDV2-vacc" in comparison to rabbits vaccinated with "convRHDV2-vacc" once. (A) Long term observation over 6 months d 0 + d 21 = vaccination; d 203 = challenge after 6 month observation; d 217 = end of trial. (B) Long-term observation over 14 months d 0 + d 21 = vaccination; d 427 = challenge after 14 month observation; d 441 = end of trial. Note: One rabbit which received a prime-boost vaccination did not have any RHDV2 specific antibody titers at day 427 before challenge and died after challenge with RHDV2 (†: death of one 2x "recRHDV2-vacc" vaccinated rabbit).

Table 5
Clinical outcome, viral load in livers and anti-RHDV2 antibody titers in sera of rabbits after heterologous challenge with RHDV1 or homologous challenge with RHDV2 after vaccination with "recRHDV2-vacc" in comparison with "convRHDV2-vacc" and non-vaccinated rabbits.

Challenge with RHDV2							
Group	surv/died	mst, h	RNA, q-RT-PCR; 2^e	viral particle, HA, 2^e	VP60, ELISA, OD	Antibody titers	
						d0	d14
recRHDV2-vacc	4/0	336	0	0	0.05	1:50	1:288
convRHDV2-vacc	4/0	336	0	0	0.06	1:50	1:1750
non-vacc	0/4	52	31.9	11	1.33	1:50	1:50
Challenge with RHDV1							
Group	surv/died	mst, h	RNA, q-RT-PCR; 2^e	viral particle, HA, 2^e	VP60, ELISA, OD	Antibody titers	
						d0	d14
recRHDV2-vacc	2/2	336/48	9.5/32.3	0/12.5	0.07/1.72	1:50	1:69
convRHDV2-vacc	3/1	336/42	8.1/33.1	0/13	0.07/1.61	1:50	1:369
non-vacc	1/3	336/45	8.8/32.5	0/12	0.06/1.4	1:50	1:50

d 0: day of vaccination; d 14: day of challenge.

3.6. A limited cross-protection against RHDV1 challenge was induced by a single vaccination with "recRHDV2-vacc"

All four rabbits vaccinated either with "recRHDV2-vacc" or with the conventional "convRHDV2-vacc" survived the homologous challenge with RHDV2 and developed no clinical signs or pathological alterations in inner organs. In contrast, only two rabbits of the "recRHDV2-vacc" group and three rabbits of the "convRHDV2-vacc" group survived the heterologous challenge infection with RHDV1. Non-vaccinated rabbits died within 90 h after RHDV2 infection or within 52 h after RHDV1 infection (three of four rabbits; Table 5) and displayed the typical diffuse necrotizing hepatitis.

In contrast, only one of the 14 survivors showed a multifocal necrotizing and granulomatous hepatitis with multinucleated giant cells, fibrosis and regenerative nodular hyperplasia.

After challenge with RHDV2 no viral load was measured, whereas after RHDV1 challenge viral RNA was detected in all livers of vaccinated rabbits but no viral particles or viral VP1 (Table 5).

4. Discussion

A new RHDV virus variant (RHDV2) emerged causing significant losses even in RHDV1 vaccinated rabbits and wild populations resulting in trials to develop RHDV2 specific vaccines [19,4]. Because RHDV cannot be cultivated in cell culture most RHDV vaccines were produced from livers of infected rabbits [2] which is very critical in regards to animal welfare. Therefore, new RHDV vaccines were developed using the RHDV capsid protein VP1 expressed in different vector systems [18,25,10,9,21].

In the present study the protective capacity of a recombinant baculovirus vaccine expressing RHDV2-VP1 was analyzed in regards to onset and duration of immunity against RHDV2 as well as to potential cross protection against RHDV1 infection. Moreover, the induction of specific anti-RHDV2 serum antibodies and the stimulation of T-cell subpopulations were investigated.

The severe character of RHD after infection with RHDV2 was proven by the typical short mean survival time after infection, the severe histopathological alterations in inner organs, high viral loads in the liver and depletion of leukocytes as described for RHDV1 or RHDV2 in non-vaccinated rabbits [29,12,1,19]. The necrotizing, acutely fatal hepatitis without relevant leukocytic infiltration is suggested to be the result of the very quick disease course and the concurrent massive immunosuppression. Importantly, the rapid onset of fever above 39 °C and the severe depletion of blood leukocytes clearly indicate the presence of a systemic inflammatory response syndrome (SIRS, [33]).

The morphological hallmark of immunosuppression is the fibrinonecrotizing splenitis with lymphatic necrosis, apoptosis and depletion after initial increase of both $CD4^+$ and $CD8^+$ T-cells in non-vaccinated rabbits and a variable degree of congestion. The survival time is too short to expect a protective antibody response [11,12] indicating the impact of RHDV induced pathological processes on T-cells and their possible involvement in resistance in naive, surviving rabbits. The depletion of blood leucocytes associated by a severe breakdown of splenic white pulp and by many apoptotic bodies in the remaining lymphatic areas supports the suggestion that T-cell depletion is one reason of the rapid fatal progress with high mortality after RHDV infection in naive rabbits [12]. The lymphohistiocytic periportal hepatitis with fewer and variable amounts of heterophils and multinucleated giant cells in 2 of the 3 non-vaccinated survivors was characterized as resorptive sequelae to previous hepatocellular damage. The higher frequency of periportal lymphohistiocytic infiltrations in the livers of surviving rabbits indicates that a cellular immune response in the liver protects the rabbits from acute lethal necrotizing hepatitis and proofs earlier findings in rabbits surviving RHDV1 infection which had increased interferon (IFN)γ levels in the liver (own data, not shown) The increased frequency of lymphatic hyperplasia in the spleen and the increased $CD4^+$ and $CD8^+$ T-cells as a normal immunological response to antigenic stimulation proofs the immunological competence of the surviving rabbits. Finally, an early activation of B- and T-cells and macrophages resulting in high expressed pro-inflammatory cytokines, similar to juvenile RHDV1 resistance [11,23] could have been induced in naive rabbits surviving the RHDV2 challenge infection. This would explain the very low HA titers, indicating a blocked RHDV2 replication. The potency of the newly established "recRHDV2-vacc" was demonstrated by the fact that even the rabbits vaccinated with the lowest dose of 256 HU developed high titers of RHDV2 VP1 specific antibodies and survived the challenge infection without clinical symptoms and detectable viral replication in liver. This correlates with previous studies where rabbits vaccinated with low doses of either inactivated RHDV virus or recombinant VP1 [2,32,18,25] survived following challenge infections. Interestingly, the induced titers of RHDV2-VP1 specific antibodies did not correlate directly with the used vaccine dose as reported for RHDV1-VP1 [22,28].

The increased number of $CD4^+$ and $CD8^+$ blood T-cells in rabbits shortly after immunization indicated that also the cellular immune response was stimulated. This strong $CD8^+$ T-cell activation by the recombinant RHDV2 vaccine could be an advantage for an early effective protection against RHDV2, because surviving non-vaccinated rabbits display also a very strong $CD4^+$ and $CD8^+$ T-cell activation. T-cell activation was confirmed before for

recombinant RHDV1-VP1 vaccine candidates as well as for liver-derived vaccines [15].

The survival of all vaccinated rabbits seven days after vaccination indicated that early protective immunity had been induced by "recRHDV2-vacc". Furthermore, because no clinical signs were found in vaccinated rabbits after challenge infection in contrast to non-vaccinated rabbits, the induced immunity seems to inhibit the productive infection of RHDV at this early time point. Such early protection was reported for a recombinant baculovirus-derived RHDV1-VP1 vaccine as already five days after a single vaccination most rabbits were protected against RHDV1 infection [18].

Conventional RHDV1 vaccines induce a protective humoral immune response from day four after vaccination which is claimed to be effective enough to protect rabbits from illness and death [2,32]. The only low antibody titers early after vaccination indicate an involvement of other early immune mechanisms. An induction of IFN-γ and IL-4 has been shown as soon at seven days post vaccination with RHDV1-VP1-VLPs and liver derived RHDV1 vaccines [15].

An interferon induction after vaccination with baculovirus alone has been discussed [14]. However, the fatal outcome of RHDV challenge infection of rabbits vaccinated with "recbacGFP-vacc" in this study does not indicate any influence of such baculovirus induced IFN on resistance against RHDV.

The survival of all vaccinated rabbits six months after vaccination with the "recRHDV2-vacc" vaccine proofed the induced long lasting immunity and the complete block of viral replication because no indications for viral replication were found. The fact that one rabbit had no detectable RHDV2 specific antibody titers six months after prime-boost vaccination, but survived a RHDV2 infection without clinical symptoms could be a result of a quick activation of memory B-cells or based on memory $CD8^+$ T-cells followed by strongly elevated IFNγ levels [36].

The completely different outcome after challenge 14 months after vaccination, where all prime-boost vaccinated rabbits displayed mild to severe clinical signs and one rabbit died indicated that the boost vaccination influenced the development of antibody titers or the formation of long-lasting B-cell memory. This seems to be a specific feature for RHDV vaccines [31,35] seen by an early decrease anti-RHDV-antibody titers already three months after vaccination [2] or no increase of RHDV specific antibodies in rabbits boostered three weeks after the first immunization with recombinant RHDV1 vaccines [8,9].

In contrast, a very effective humoral immune response was induced by a RHDV infection already after seven days post prime vaccination which confirmed earlier studies with recombinant vaccines [28]. A strong and reliable cellular immunity that convey life-long protection against RHD is also seen in naive rabbits that survived a RHDV infection [27,11,23].

The cross protection against RHDV1 induced by single vaccination with "recRHDV2-vacc" or "convRHDV2-vacc" is limited as found after vaccination with conventional anti-RHDV1 vaccine "Cunivak RHD", with only a partial cross-protection against RHDV2. However, a prime-boost vaccination with "Cunivak RHD" conveyed full cross-protection against RHDV2 [24]. The molecular basis of a possible cross-protection was identified in seven hypervariable regions distributed over the VP1 protein where possible cross-reactive epitopes were found using specific monoclonal antibodies against different RHDV variants [19]. Cross-protectivity is known between different RHDV variants as the non-pathogenic Australian strain RCV-A1 induced partial cross-protection in rabbits against the virulent Czech RHDV1 strain V351 which was released to decimate rabbit population [34]. But also a break of immunity in wild rabbits surviving an RHDV1 (strain V351) infection by the RHDV1 strain K5 was reported (pestsmart.com). This ongoing adaptation of RHDV will result most likely in new RHDV variants in the future underlining the need to constantly adapt RHDV vaccines.

In conclusion, this study confirms that the newly established recombinant vaccine based on RHDV2-VP1 not only protects rabbits after a single vaccination against RHD disease but also reduces viral replication to low level and therefore restrict viral shedding. The ability of the vaccine to protect rabbits younger than 8 weeks requires further examination because the juvenile resistance or maternal anti-RHDV might interfere with the vaccination inducible immunity against RHDV2.

Acknowledgements

The authors thank Günther Keil, Sabine Weber, Gabriele Czerwinski, Ralf Henkel and Thomas Pieper for their excellent support.

This work was funded by IDT Biologika Insel Riems.

Conflict of interest

The authors declare no conflict of interest.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vaccine.2019.04.061.

References

[1] Abrantes J, van der Loo W, Le Pendu J, Esteves PJ. Rabbit haemorrhagic disease (RHD) and rabbit haemorrhagic disease virus (RHDV): a review. Vet Res 2012;43:12.
[2] Argüello Villares JL. Viral haemorrhagic disease of rabbits: vaccination and immune response. Rev Sci Tech 1991;10(2):459–80.
[3] Baily JL, Dagleish MP, Graham M, Maley M, Rocchi MS. RHDV variant 2 presence detected in Scotland. Vet Rec 2014;174(16):411.
[4] Bárcena J, Guerra B, Angulo I, González J, Valcárcel F, Mata CP, et al. Comparative analysis of rabbit hemorrhagic disease virus (RHDV) and new RHDV2 virus antigenicity, using specific virus-like particles. Vet Res 2015;46:106.
[5] Camarda A, Pugliese N, Cavadini P, Circella E, Capucci L, Caroli A, et al. Detection of the new emerging rabbit haemorrhagic disease type 2 virus (RHDV2) in Sicily from rabbit (Oryctolagus cuniculus) and Italian hare (Lepus corsicanus). Res Vet Sci 2014;97(3):642–5.
[6] Duarte M, Carvalho C, Bernardo S, Barros SV, Benevides S, Flor L, et al. Rabbit haemorrhagic disease virus 2 (RHDV2) outbreak in Azores: Disclosure of common genetic markers and phylogenetic segregation within the European strains. Infect Genet Evol 2015;35:163–71.
[7] Duarte M, Henriques M, Barros SC, Fagulha T, Ramos F, Luís T, et al. Detection of RHDV variant 2 in the Azores. Vet Rec 2015;176(5):130.
[8] Farnós O, Fernández E, Chiong M, Parra F, Joglar M, Méndez L, et al. Biochemical and structural characterization of RHDV capsid protein variants produced in Pichia pastoris: advantages for immunization strategies and vaccine implementation. Antiviral Res 2009;81(1):25–36.
[9] Fernández E, Toledo JR, Chiong M, Parra F, Rodríguez E, Montero C, et al. Single dose adenovirus vectored vaccine induces a potent and long-lasting immune response against rabbit hemorrhagic disease virus after parenteral or mucosal administration. Vet Immunol Immunopathol 2011;142(3–4):179–88.
[10] Fernández-Fernández MR, Mouriño M, Rivera J, Rodríguez F, Plana-Durán J, García JA. Protection of rabbits against rabbit hemorrhagic disease virus by immunization with the VP60 protein expressed in plants with a potyvirus-based vector. Virology 2001;280(2):283–91.
[11] Ferreira PG, Costa-E-Silva A, Oliveira MJ, Monteiro E, Aguas AP. Leukocyte-hepatocyte interaction in calicivirus infection: differences between rabbits that are resistant or susceptible to rabbit haemorrhagic disease (RHD). Vet Immunol Immunopathol 2005;103(3–4):217–21.
[12] Ferreira PG, Costa-e-Silva A, Oliveira MJ, Monteiro E, Cunha EM, Aguas AP. Severe leukopenia and liver biochemistry changes in adult rabbits after calicivirus infection. Res Vet Sci 2006;80(2):218–25.
[13] Gall A, Hoffmann B, Teifke JP, Lange B, Schirrmeier H. Persistence of viral RNA in rabbits which overcome an experimental RHDV infection detected by a highly sensitive multiplex real-time RT-PCR. Vet Microbiol 2007;120(1–2):17–32.
[14] Gronowski AM, Hilbert DM, Sheehan KC, Garotta G, Schreiber RD. Baculovirus stimulates antiviral effects in mammalian cells. J Virol 1999;73(12):9944–51.
[15] Guo H, Zhu J, Tan Y, Li C, Chen Z, Sun S, et al. Self-assembly of virus-like particles of rabbit hemorrhagic disease virus capsid protein expressed in

Escherichia coli and their immunogenicity in rabbits. Antiviral Res 2016;131:85–91.
[16] Hall RN, Mahar JE, Haboury S, Stevens V, Holmes EC, Strive T. Emerging rabbit hemorrhagic disease virus 2 (RHDVb), Australia. Emerg Infect Dis 2015;21(12):2276–8.
[17] Hoffmann B, Depner K, Schirrmeier H, Beer M. A universal heterologous internal control system for duplex real-time RT-PCR assays used in a detection system for pestiviruses. J Virol Methods 2006;136(1–2):200–9.
[18] Laurent S, Vautherot JF, Madelaine MF, Le Gall G, Rasschaert D. Recombinant rabbit hemorrhagic disease virus capsid protein expressed in baculovirus self-assembles into viruslike particles and induces protection. J Virol 1994;68(10):6794–8.
[19] Le Gall-Reculé G, Lavazza A, Marchandeau S, Bertagnoli S, Zwingelstein F, Cavadini P, et al. Emergence of a new lagovirus related to Rabbit Haemorrhagic Disease Virus. Vet Res 2013;44:81.
[20] Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(T)(-Delta Delta C) method. Methods 2001;25(4):402–8.
[21] López-Vidal J, Gómez-Sebastián S, Bárcena J, Nuñez Mdel C, Martínez-Alonso D, Dudognon B, et al. Improved production efficiency of virus-like particles by the baculovirus expression vector system. PLoS ONE 2015;10(10):e0140039.
[22] Marín MS, Martín Alonso JM. Pérez Ordoyo García LI, Boga JA, Argüello-Villares JL, et al.: Immunogenic properties of rabbit haemorrhagic disease virus structural protein VP60 expressed by a recombinant baculovirus: an efficient vaccine. Virus Res 1995;39(2–3):119–28.
[23] Marques RM, Costa-E-Silva A, Águas AP, Teixeira L, Ferreira PG. Early inflammatory response of young rabbits attending natural resistance to calicivirus (RHDV) infection. Vet Immunol Immunopathol 2012;150(3–4):181–8.
[24] Müller C, Ulrich R, Franzke K, Müller M, Köllner B. Crude extracts of recombinant baculovirus expressing rabbit hemorrhagic disease virus 2 VLPs from both insect and rabbit cells protect rabbits from rabbit hemorrhagic disease caused by RHDV2. Arch Virol 2019;164(1):137–48.
[25] Nagesha HS, Wang LF, Hyatt AD, Morrissy CJ, Lenghaus C, Westbury HA. Self-assembly, antigenicity, and immunogenicity of the rabbit haemorrhagic disease virus (Czechoslovakian strain V-351) capsid protein expressed in baculovirus. Arch Virol 1995;140(6):1095–108.
[26] Ohlinger VF, Haas B, Thiel HJ. Rabbit hemorrhagic disease (RHD): characterization of the causative calicivirus. Vet Res 1993;24(2):103–16.
[27] Patton NM. Viral hemorrhagic disease. A major new disease problem of rabbits. Rabbit Res 1989;12:64–7.
[28] Plana-Duran J, Bastons M, Rodriguez MJ, Climent I, Cortés E, Vela C, et al. Oral immunization of rabbits with VP60 particles confers protection against rabbit hemorrhagic disease. Arch Virol 1996;141(8):1423–36.
[29] Prieto JM, Fernández F, Alvarez V, Espi A, García Marín JF, Alvarez M, et al. Immunohistochemical localisation of rabbit haemorrhagic disease virus VP-60 antigen in early infection of young and adult rabbits. Res Vet Sci 2000;68(2):181–7.
[30] Puggioni G, Cavadini P, Maestrale C, Scivoli R, Botti G, Ligios C, et al. The new French 2010 Rabbit Hemorrhagic Disease Virus causes an RHD-like disease in the Sardinian Cape hare (Lepus capensis mediterraneus). Vet Res 2013;44:96.
[31] Radaelli A, Zanotto C, Perletti G, Elli V, Vicenzi E, Poli G, et al. Comparative analysis of immune responses and cytokine profiles elicited in rabbits by the combined use of recombinant fowlpox viruses, plasmids and virus-like particles in prime-boost vaccination protocols against SHIV. Vaccine 2003;21(17–18):2052–64.
[32] Smíd B, Valícek L, Rodák L, Stěpánek J, Jurák E. Rabbit haemorrhagic disease: an investigation of some properties of the virus and evaluation of an inactivated vaccine. Vet Microbiol 1991;26(1–2):77–85.
[33] Stearns-Kurosawa DJ, Osuchowski MF, Valentine C, Kurosawa S, Remick DG. The pathogenesis of sepsis. Annu Rev Pathol. 2011;6:19–48. 10.1146.
[34] Strive T, Wright JD, Robinson AJ. Identification and partial characterisation of a new Lagovirus in Australian wild rabbits. Virology 2009;384(1):97–105.
[35] Vaine M, Wang S, Crooks ET, Jiang P, Montefiori DC, Binley J, et al. Improved induction of antibodies against key neutralizing epitopes by human immunodeficiency virus type 1 gp120 DNA prime-protein boost vaccination compared to gp120 protein-only vaccination. J Virol 2008;82(15):7369–78.
[36] West DJ, Calandra GB. Vaccine induced immunologic memory for hepatitis B surface antigen: implications for policy on booster vaccination. Vaccine 1996;14(11):1019–27.
[37] Westcott DG, Frossard JP, Everest D, Dastjerdi A, Duff JP, Steinbach F, et al. Incursion of RHDV2-like variant in Great Britain. Vet Rec 2014;174(13):333.
[38] Xu ZJ, Chen WX. Viral haemorrhagic disease in rabbits: a review. Vet Res Commun 1989;13(3):205–12.