VETBIONET

Veterinary Biocontained facility Network for excellence in animal infectiology research and experimentation

Deliverable D8.1

Development of a differential serological assay to separate RHDV infection from RHDV2

Due date of deliverable: M24

Actual submission date: M37

Start date of the project: March 1st, 2017 **Duration:** 60 months

Organisation name of lead contractor: INIA

Revision: V2

Dissemination level	
Public	**X**
Confidential, only for members of the consortium (including Commission Services)	
Classified, as referred to in Commission Decision 2001/844/EC	

Summary

Objectives:

The main aim of this deliverable is to improve the diagnostic tools and methods currently available for the detection of RHDV, in order to differentiate emerging strains causing economic impact as RHDV2 (GI.2).

Rationale:

INGENASA has broad experience in commercialization and manufacturing of diagnostic products in the Animal Health sector and currently has two products for RHDV in the market. The improvement of such diagnostic systems by development of new one able to detect specifically RHDV2 has been addressed in this deliverable. This can be done by generating monoclonal antibodies specific to RHDV GI.2, based on the differential antigenic properties exhibited by this virus compared to RHDV GI.1, as well as by the use of recombinant purified virus-like particles (VLPs) that are morphologically and antigenically identical to infectious RHDV virions.

Teams involved:

INGENASA

INIA

ANSES

Introduction

Rabbit hemorrhagic disease virus (RHDV) is the prototype species of the Lagovirus genus within the Caliciviridae family (non-enveloped, icosahedral, single-stranded positive-sense RNA viruses). This genus comprises viruses causing severe diseases in the European rabbit (Oryctolagus cuniculus) and in several hare species (Lepus spp.). The disease caused by RHDV is highly contagious and usually fatal in adult rabbits. It was reported in China in 1984, and spread rapidly around the world, being currently enzootic in wild rabbit populations in Europe, North Africa, Australia and New Zealand. When it emerged, RHD dramatically reduced wild rabbit populations and was responsible for great economic losses in the rabbit industry worldwide. Efficient inactivated vaccines introduced in the early 1990s, together with other control measures enabled the gradual control of RHD in rabbitries for more than 20 years. However, in 2010 a new RHDV related virus (named RHDV2 or more recently GI.2) with a distinctive pathogenic profile, emerged in France with an unknown origin, rapidly spreading worldwide throughout domestic and wild rabbit populations.

Thus, currently, the impact of RHDV GI.2 in the rabbit industry, wild rabbit populations and consequent implications for endangered species conservation (i.e. Iberian lynx and Spanish imperial eagle) are a cause of major concern.

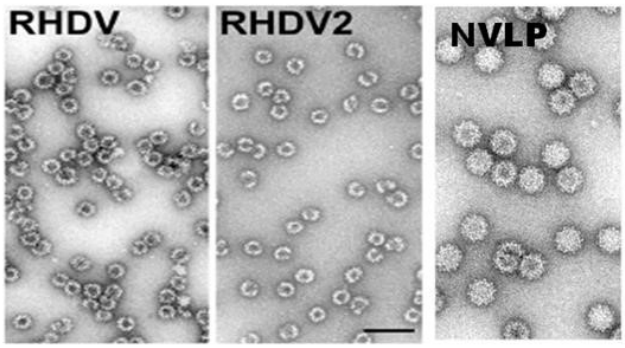
This epidemiological situation of emergence of new RHDV give rise to the urgent need of develop new diagnostic tools to design assays for efficient disease monitoring.

Result

3.1 Generation of RHDV diagnostic tools; recombinant RHDV-VLPs and specific rabbit polyclonal sera.

At INIA, we have generated and characterised RHDV GI.1 (RHDV1), RHDV GI.2 (RHDV2) and RHDV GI.3 RCV-E1 (RHDV-NVL) specific VLPs. Firstly we generated recombinant baculoviruses expressing the VP60 protein of that RHDV strains. The three VP60 proteins were expressed to roughly similar levels in infected H5 insect cells (not shown). To determine the correct assemble of VP60 protein into particulate material (VLPs), infected-cell extracts were subjected to a VLP-purification protocol developed in our lab at INIA. Electron microscopy analyses of negatively stained preparations showed that the three VP60 proteins assembled with similar efficiency into VLPs of approximately 40 n, in diameter (Figure 1).

Figure 1. - Electron micrographs of negatively stained samples of the RHDV GI.1, RHDV GI.2 and RHDV non-pathogenic GI.3 virus-like-particles (VLPs)



In order to develop specific assays for RHDV GI.2 detection, the recombinant baculovirus expressing the capsid protein from this RHDV strain was transfer to INGENASA (MTA signed).

In addition, purified VLPs of the three RHDV strains were transfer to ANSES for generation of specific rabbit polyclonal antibodies. The rabbit immunization at ANSES BSL2 facilities was conducted according to biosafety and bioethical procedures (ComEth Anses/ENVA/UPEC ethical committee agreement number 16).

For this purpose, eleven 12-week-old EOPS New Zealand White rabbits were used. They were split into two groups: 1) two rabbits as control and 2) three groups of three rabbits inoculated with one type of VLPs. Daily observations for morbidity and mortality were performed. During the trial, one inoculated rabbit died without apparent cause at D24 and one injured control rabbit was killed for humanely considerations. At the end of the experimental trial (day 42), surviving animals were humanely killed and examined for macroscopic lesions.

Each rabbit was inoculated by the subcutaneous route in the neck region (500 µL per inoculation) at day (D) 0 (prime) then at D21 (boost). The two control rabbits were inoculated with an emulsion of 250 µL of adjuvant (Montanide 50V2, Seppic) with 250 µL of PBS, whereas each inoculated rabbits received an emulsion of 250 µL of adjuvant with 250 µL PBS containing 400 µg of VLP (three rabbits per group). Blood samples were collected at the beginning of the assay (D0), at D7, at D21 just before the boost and at D35. At the sacrifice (D42), the totality of the blood was collected. The different sera were stored at -20°C then sent to INIA at the end of the trial.

Detection and titration of antibodies of the control and immunized rabbit sera were performed by INIA using ELISA tests against the three different VLPs. ELISA results confirmed that the rabbits had no lagovirus antibody at D0. At day 7 post-immunization was already detected antibody titres against VP60 protein. This humoral response increased significantly after boost. The Figure 2 shows the end-point rabbit antibody titres against each VLP, elicited by immunization with each of the VLPs indicated in the top of the figure. The statistical differences between antibody titres against particular VLPs were detected by ANOVA analyses with post-test with Tukey (indicated in the graph by * or **).

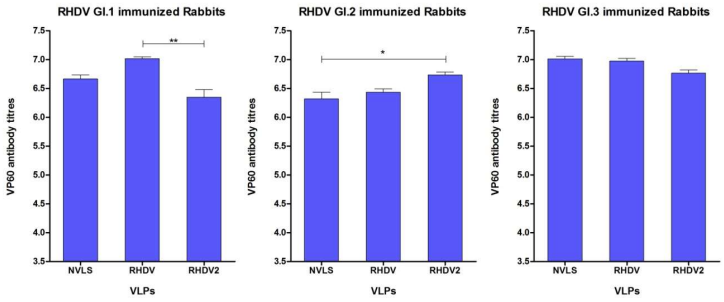


Figure 2. - VP60 antibody titres at day 42 post--immunization of sera from EOPS rabbits immunized with RHDV GI.1, GI.2 and GI.3.

These results confirm the immunogenicity of the three recombinant VLPs, their capacity to elicit high antibody titres and the potential use of such specific polyclonal rabbit sera as RHDV reference sera.

3.2 Generation of new specific monoclonal antibodies (MAbs) for specific differentiation of RHDV GI.1 and RHDV GI.2.

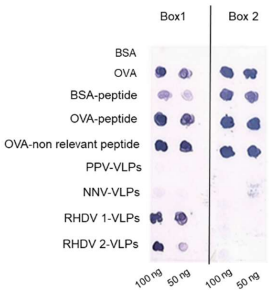
Seven hypervariable regions (V1-V7) were defined at INIA, in the protrusion (P) domain (C-terminal region) of the RHDV capsid protein. INGENASA in collaboration with INIA, designed a peptide partially specific of RHDV2 which was synthesized and conjugated to ovalbumin (OVA) and bovine serum albumin (BSA) carrier proteins (OVA-peptide and BSA-peptide, respectively). Mouse immunizations with the peptides were performed and a first fusion was carried out. Due to problems on the conjugation of the peptides, which was detected after the mouse immunization, no positive results were obtained and it was necessary to synthesize new peptides.

A second immunization was performed following two different protocols:

- Box 1: RHDV2 VLPs in the first immunization and OVA-peptide in the following immunizations
- Box 2: OVA-peptide in all immunizations

The mouse sera reactivity to RHDV2-VLPs, OVA-peptide and controls such as BSA-peptide, RHDV1-VLPs, PPV-VLPs, Nodavirus-VLPs (NNV-VLPs), OVA and BSA, was analyzed byELISA and Dot-Blot. The results are shown in Figures 3 and 4.

Figure 3. Reactivity of mouse sera by Dot Blot.



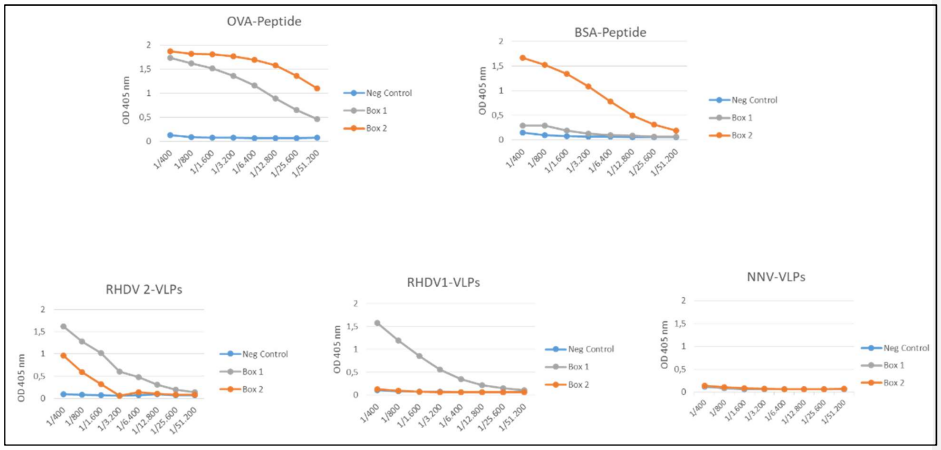


Figure 4. Reactivity of sera from mice immunized with RHDV2 specific peptide (200 ng/well) and different VLPs (RHDV1 and RHDV2 and Nodavirus VLPs, NNV, as negative control) (100 ng/well), by ELISA. The graphics represent the average reactivity of four mice. The absorbance at 450 nm was plotted against the sera dilution.

The reactivity of sera from mice immunized with BSA, OVA and non-relevant peptide conjugated with OVA was also assessed by ELISA, with the expected results (data not shown).

As shown in Fig. 3 and 4, the sera from Box 2 are able to recognize OVA- and BSA-peptides as expected; however, the sera mice from Box 1 weakly recognize BSA-peptide. On the other hand, sera from Box 1 are able to recognize RHDV2-VLPs as well as RHDV1-VLPs, as expected, but sera from Box 2, only recognize weakly RHDV2-VLPs in ELISA and not in Dot Blot.

With these results, we decided to perform two independent fusion experiments with one mouse of Box 1 and two mice from Box 2. After the selection process with the BSA-peptide, OVA and RHDV2-VLPs, 9 MAbs were selected and characterized. None of them, showed enough specificity against RHDV2-peptide or RHDV2-VLPs, to continue working with them as diagnostic tools.

The mice were boosted with the antigens, the sera checked and a new fusion was programmed.

INGENASA performed three cellular fusions, all of them with negative results.

Therefore, the remained mice were subjected to new immunizations as follows:

- Box 1:
    - First immunization: RHDV2 VLPs (50 µg)
    - 2nd-4th immunization: OVA-peptide (50 µg)
    - Additional immunizations during this period (5th-6th immunization): RHDV2 VLPs (25 µg)
- Box 2: All immunizations were carried out with OVA-peptide (50 µg)

Once more, two new independent fusion experiments were conducted, in this case with two mice of Box 1 and one mouse from Box 2.

From the fusion with the mice of the Box 1, 5 MAbs specific of RHDV2 and 5 MAbs able to recognize RHDV1 and 2, have been selected. Table 1 reports the name, specificity and isotype of the MAbs obtained.

The MAbs have been purified from culture supernatants, after growing the selected hybridoma cells *in vitro*. Only in those cases where the yield of the MAbs was too low, they were purified from ascitic fluid. Then, the isotype of each MAbs was identified and, finally, the purified MAbs have been labeled with peroxidase (HRPO), for their later use in different ELISAs. Further characterization by other techniques, such as Western Blot, immunofluorescence, immunohistochemistry will be carried out.

Table 1: Specificity and Isotype of the selected MAbs.

mAb	Specificity	Isotype
11D10	RHDV2	IgG2a

11C11	RHDV2	IgG2b
11F3	RHDV2	IgG2a
16F12	RHDV2	IgG1
14E11	RHDV2	IgG2a
16H7	RHDV1/RHDV2	IgG3
12A1	RHDV1/RHDV2	IgG2a
13A7	RHDV1/RHDV2	IgG2b
13F4	RHDV1/RHDV2	IgG2b
16A9	RHDV1/RHDV2	IgG2b

3.3 Development of different assay formats for specific detection of RHDV GI.2

The specific RHDV2 MAbs and those that recognize both RHDV1 and 2, have been tested by double antibody sandwich ELISA (DAS-ELISA) and lateral flow immunoassays, for the detection of both viruses. Preliminary results indicate that both assays could be useful tools to be used in the laboratory and in the field, respectively. The final optimization of both assays is being finalized and their diagnostic specificity and sensitivity will be evaluated.

Moreover, in order to differentiate specific antibodies against RHDV1 or 2, a competition ELISA is under development.

In order to fulfill the evaluation of the new assays, well characterized field and experimental samples will be tested. Those samples will be provided by INIA and other partners of the Consortium.

The development of a lateral flow assay (LFA) for the differential detection of RHDV 1 and 2 was performed. First, all the Ingenasa-produced MAbs were tested, analyzing all the possible combinations between them. Promising results were only obtained when using two specific combinations:

1. 2E11 as capturing and as detection reagent, for the detection of both RHDV 1 and 2.

(2E11 is a MAb that was already available in Ingenasa before the start of this project. It detects both types of RHDV).

2. 16F12 as capturing and 2E11 as detection agent, for the specific detection of RHDV 2.

The initial design of the test was:

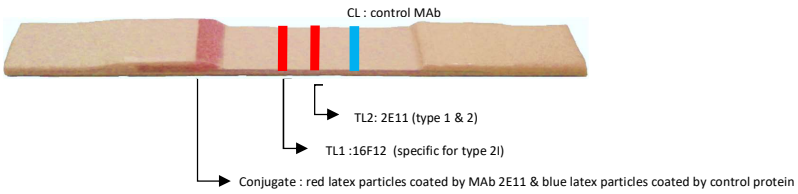


Figure 5. Prototype of laminar flow for differential detection of RHDV 1 and RHDV 2

If the sample contains RHDV 1 or 2, conjugated MAb 2E11 attached to red latex particles will bind to the viral VP60 and the complex will start the migration through the strip. Once the VP60-MAb-latex complex reaches the test lines (TL), capturing MAbs contained in TL will also bind to VP60, stopping the migration of the particles and leading to the appearance of a red line. Two red lines will appear if the sample contains RHDV 2, whereas only the upper red line will appear if the sample contains RHDV 1. The blue control line must always appear; if not, the test must be considered invalid.

As a proof of concept, RHDV2-VLPs, RHDV1-VLPs and NVL-VLPs were tested. Positive VLPs were diluted from 5 to 0.04 µg/mL in two different running buffers (RB), to test not only sensitivity, but also the buffer composition (Table 2).

Commenté [LG1]: You have proposed his name page 5 at the beginning of result chapter to design non-pathogenic rabbit lagoviruses

Table 2: Optimisation of lateral flow assay for RHDV detection: testing sensitivity and type of buffer.

VLPs/RB		5 µg/ml	2.5	1.25	0.62	0.31	0.16	0.08	0.04	0
RHDV 1 / RB1	TL2	+	+	+	+	+	±	δ	neg	neg

	TL 1	neg	neg	neg	neg	neg	neg	neg	neg	neg
RHDV 1 / RB2	TL 2	+	+	+	+	±	δ	s	neg	neg
	TL 1	neg	neg	neg	neg	neg	neg	neg	neg	neg
RHDV 2 / RB1	TL 2	+	+	+	+	+	+	±	δ	neg
	TL 1	+	+	+	+	+	±	δ	s	neg
RHDV 2 / RB2	TL 2	+	+	+	+	+	+	±	δ	neg
	TL 1	+	+	+	+	+	±	δ	s	neg

Control lines were always intense and homogeneous. NVL-VLPs (RHDV non pathogenic strain) were not detected, no red lines were observed. Regarding positive VLPs, the performances with RB1 and RB2 were quite similar, but RB2 was chosen since it contains a detergent that could be useful when testing field samples. Sensitivity was close to 0.16 µg/mL of RHDV 1/2-VLPs in all cases.

As a following step, several field samples that were already available at Ingenasa (7 negative, 1 positive to RHDV 1) were analysed following the next protocol:

-Weigh 1g of liver and mash it using a bistoury or the bottom of a sterile syringe

-Add 2 mL of PBS buffer

-Vortex 30''- 1'

-Centrifuge 4500 x g for 5'

-Keep the supernatant (freeze it at -80 ºC if it is not going to be used in that same moment)

For the analysis, 10 µL of the sample were added to the sample pad of the strip, followed by 110 µL of RB2. All negative samples led to a negative result, whereas the positive one was detected as positive for RHDV 1 (only upper red line appeared). However, background appeared in TL1.

Then, the extraction of one negative liver was spiked with decreasing concentrations of RHDV1 / RHDV2 VLPs, to study the matrix effect in the test performance. Sensitivity decreased to 1.25 µg/mL of RHDV 1-VLPs and to 0.62 µg/mL of RHDV 2-VLPs. Again, background appeared in TL1.

In addition to these analyses, the LFA prototype was used to test livers homogenized from rabbits infected with the RHDV strains showed in the table 2, provided by ANSES.

Strain	Gentoype	Concentration
99-05	RHDV G6 (GI.1a)	1.3E+8 copies/µL
11-85	RHDV G1 (GI.1b)	1.1E+9 copies/µL
16-09	RHDV G5 (GI.1d)	1.4E+8 copies/µL
17-09	RHDV2 (GI.2)	2.8E+8 copies/µL

For the analyses 10 µL of each sample were added to the sample pad of the strip, followed by 110 µL of chromatography buffer (final dilution 1/12). Sample serial dilutions were made until signal was lost. The figure 6 shows the results achieved. The sensitivity for detection of RHDV genotype GI.2 is higher than for detection of GI.1.

When the quantity of RHDV2 in the samples is dropped, the intermediate signal line is lost and only the bottom line is visible, since all the viral particles are retained in the first line. This fact should be taken into account for the interpretation of the results.

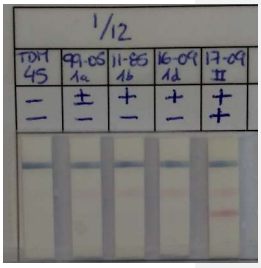
Optimization of this assay is ongoing and the analyses of field samples is planned to be performed along this year 2020.

Positive	+
Weak Positive	±
Doubtful	δ
Negative	s/neg

STRAIN 99-05, Gentoype RHDV G6 (GI.1a)		Dilution	
		1/12 (1.1E+7 copies/µl)	1/48 (2,7E+6 copies/µL)
Result in LFA	Test line (1 & 2 detection)	±	neg
	Test line (specific 2 detection)	neg	neg

STRAIN 11/85, Gentoype RHDV G1 (GI.1b)		Dilution	
		1/12 (9,2E+7 copies/µL)	1/48 (2,3E+7 copies/µL)
Result in LFA	Test line (1 & 2 detection)	+	neg
	Test line (specific 2 detection)	neg	neg

STRAIN 16/09, Gentoype RHDV G5 (GI.1d)		Dilution	
		1/12 (1,2E+7 copies/µL)	1/48 (2,9 E+6 copies/µL)
Result in LFA	Test line (1 & 2 detection)	+	δ±
	Test line (specific 2 detection)	neg	neg



STRAIN 17/09, Gentoype RHDV2 (GI.2)		Dilution				
		1/12 (2,3E+7 copies/µL)	1/48 (5,8 E+7 copies/µL)	1/96 (2,9 E+7 copies/µL)	1/192 (1,5 E+7 copies/µL)	1/384 (7,3 E+5 copies/µL)
Result in LFA	Test line (1 & 2 detection)	+	δ	δ	S	neg
	Test line (specific 2 detection)	+	+	+	±	δ±

Figure 6. Analyses of rabbit liver homogenates infected with RHDV by prototype of laminar flow developed for differential detection of RHDV 1 and RHDV 2

4. Conclusions

RHDV diagnostic tools have been developed and characterized. These include the generation of: recombinant VLPs (to be used as antigens in the different immunoassays), specific rabbit polyclonal sera against different RHDV types (to be used as reference sera) and MAbs that specifically recognize both RHDV1 and RHDV2 or that are specific to RHDV2. These MAbs are quite relevant since they allow differentiating between RHDV1 and RHDV2 genotypes.

In addition, different format assays have been designed and preliminary evaluated, including a double antibody sandwich ELISA (DAS-ELISA), a Lateral Laminar Flow (LF) and a competitive ELISA (this assay being currently under evaluation).