EFFECT OF VACCINATION ON PROTECTION AGAINST *RHDV-2* AND VIRAL LOAD

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ABSTRACT

Vaccination against Rabbit haemorrhagic disease (RHD) is the principal measure available for protection against this lethal virus, although limited scientific information is available about the impact of vaccination on disease replication and spread. The aim of this study was to assess the clinical course, viral load, survival rate and humoral immune response of animals vaccinated with ERAVAC after experimental RHDV2 infection at 6 months post-vaccination. These analyses may lead to a better understanding of the effect of vaccination on RHDV2 transmission in the long term. To this end, 38 New Zealand rabbits of 1 month of age were randomly distributed between two groups of equal size; the first group was vaccinated with ERAVAC (V group) and the second group received PBS (C group - control). After 6 months, control and vaccinated rabbits were challenged with a heterologous virulent RHDV2 strain and clinically monitored for 7 days. All the animals were necropsied and blood, organs and faeces were sampled for detection of the viral load. The results showed that vaccination with ERAVAC provides full protection against mortality after experimental challenge and prevents the spread of RHDV in faeces, as well as the persistence of the virus in major target organs, in RHDV2 infected adult rabbits at 6 months post-vaccination. This study contributed to describing the effect of the vaccine on RHDV2 transmission, being the main alternative for RHDV2 control on farms.

Key words: Rabbit haemorrhagic disease, vaccine, RHDV2 prevention, efficacy, RT-qPCR.

INTRODUCTION

Rabbit haemorrhagic disease (RHD) is one of the most important fatal diseases in the rabbit industry, which induces significant economic losses. Vaccination has been one of the main prevention measures for avoidance of these catastrophic consequences. Therefore, after the emergence of the type 2 variant virus (RHDV2 or GI.2) in 2010, inactivated vaccines were developed and marketed in the European Union member states (Valls *et al*. 2016). This new variant of the virus is characterized by spreading worldwide within a short period of time and showing higher prevalence than classical RHDV isolates in kits and adult rabbits. Direct contact between rabbits and fomites plays an important role in farm outbreaks. Recently, Dalton *et al*. 2018 identified the faecal route as the main source of RHDV2 dissemination. The vaccination strategy has allowed the control of RHDV2 disease on farms, showing early protection against mortality and clinical signs after experimental challenges (Montbrau *et al.* 2016, Le Minor *et al.* 2019). However, no data are available about its effects on RHDV2 replication and spread in the long term.

The aim of this study was to assess the effects of an inactivated vaccine on the clinical course, viral load, survival rate and humoral immune response in experimentally infected adult rabbits at 6 months post-vaccination (mpv). Such analysis may lead to a better understanding of the effect of the vaccine on RHDV2 transmission in the long term.

MATERIALS AND METHODS

Animals, experimental design and challenge infection

Thirty-eight one-month-old New Zealand White rabbits, free of major rabbit diseases including RHD, were selected. At the experimental facilities, all the animals were clinically examined and the absence

of antibodies against RHDV2 was verified. They were then ear-tagged and randomly divided into two groups: Vaccinated (V group; n = 19) and Control (C group; n = 19). After acclimatization, the animals in V group were vaccinated subcutaneously with 0.5 mL ERAVAC vaccine following the manufacturer's recommendations (day 0). Rabbits in C group were immunized with 0.5 ml sterile PBS.

At 6 mpv, the animals were challenged intramuscularly with a heterologous virulent RHDV2 strain ''V-1035" (1000 hemagglutination units). After the challenge, the health status of all the rabbits was monitored over 7 days. Blood and faeces were sampled as described below until the rabbits were euthanized in a moribund state or died. Seven days after challenge, all remaining animals were euthanized, and blood and organ samples were taken. All procedures involving animals were conducted following the European Union Guidelines for Animal Welfare (Directive 2010/63/UE) and approved by the Ethics Committee of HIPRA Scientific SLU.

Sampling and necropsy
Blood samples were collected monthly from vaccination until the challenge. After the challenge, blood samples were collected at 0 and 7 days, whilst faecal samples were collected on days 0, 2, 4 and 7 post-challenge from 10 rabbits per group. Furthermore, all rabbits underwent necropsy, and liver and spleen samples were collected, properly referenced and stored.

Laboratory analysis: Humoral response and evaluation of RHDV viral load in liver, spleen and faeces
Sera were tested to detect and quantify the antibody response against RHDV2 by using an *in-house* competition ELISA (cELISA) (OIE, 2010). Neutralization titres were expressed as the Log_{10} of the reciprocal titre. Values ≥1:10 (Log_{10}=1) were considered to have biological significance.

Quantification of the RHDV2 viral load in liver, spleen and faeces was determined by real-time RT-qPCR (Duarte *et al.* 2015). RNA was purified from the samples using the RNeasy Mini Kit (Qiagen, Germany) according to the manufacturer's protocol. The viral load in different samples after RHDV2 infection was determined using 35 ng of purified RNA by qRT-PCR method using the QuantiTect Probe RT-PCR Kit (Qiagen, Germany). Nuclease-free water served as the non-template control and standard RHDV2-RNA with 4.82 x 10^{11} copies/µl served as the positive control. Calculations were performed using ''7500 Software version 2.0.6" where the sample Ct values were used to estimate template quantity by comparing them to the standard curve. Viral load was calculated as viral RNA copies/mg faeces, expressed as Log10.

Statistical analysis
Statistical comparison was performed by SPSS® using Kruskal-Wallis, Mann-Whitney U test, Student's T-test or Fisher's test with a significance level of 95%.

RESULTS AND DISCUSSION

Dynamics of humoral immune response pre-challenge.
All the rabbits were confirmed to be free of antibodies against RHDV2 prior to vaccination. In addition, the control group remained seronegative throughout the post-vaccination period, whilst the vaccinated animals responded serologically to vaccination at 7 days post-vaccination (dpv) and remained seropositive over the study period (Figure 1). Vaccinated animals showed an average of 1.35±0.41 Log_{10} antibody titre at 7 dpv; increasing to the peak of humoral response at 3 mpv (2.37±0.46 Log_{10} antibody titre). From 1 mpv to 6 mpv, the rabbits showed similar average titres ranging between 2.05 and 2.37 Log_{10} antibody titre.

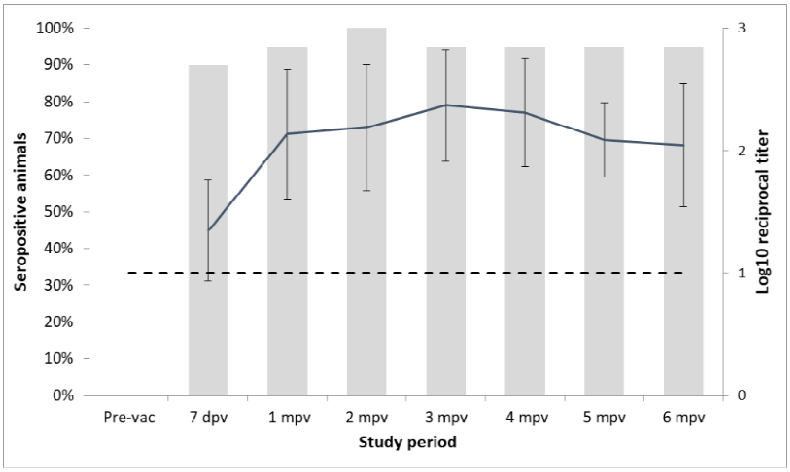

Figure 1: Percentage of seropositive rabbits (bars) and the geometric mean of antibody titres of seropositive animals (line) following RHDV-2 vaccination. Antibody titres are expressed as the reciprocal antibody titre, expressed as Log10. Sera with titres higher than 1/10 were considered to be of biological significance (black dotted line).

***RHDV2* challenge at 6 months post-vaccination: clinical course, survival rate and humoral immune response**

All the rabbits vaccinated with ERAVAC survived after RHDV2 challenge infection and did not show any RHD-specific clinical symptoms, except 1 rabbit with clinical symptoms at 1 day post-challenge (dpc). However, the unvaccinated control group showed a 47.37% mortality and an average survival time post-challenge of 4.47±0.61 days. This result shows the severe character of RHDV2 after infection by the typical short mean survival time for RHD in non-vaccinated rabbits (Le Gall-Reculé *et al.* 2013). Furthermore, we also recorded clinical signs of RHD in 42.10% of rabbits in the control group from 1 to 2 dpc. The typical signs of RHD were marked depression with dyspnoea in some cases.

After challenge, similar antibody response profiles were observed in the vaccinated and control groups, with a significant increase in both groups at 7 dpc (U Mann-Whitney test; $p<0.0001$), this was also significantly higher in the vaccinated than in the non-vaccinated rabbits on that day (U Mann-Whitney test; $p<0.0001$).

***RHDV2* challenge at 6 months post-vaccination: evaluation of RHDV viral load.**

To assess the impact of vaccination on disease replication and spread, the mean quantitative viral load of vaccinated infected animals was compared with that obtained from non-vaccinated infected rabbits in the liver, spleen and faeces.

Table 1: Percentage of RNA-positive rabbits and the geometric mean of viral load of RNA-positive animals in liver and spleen following RHDV2 challenge at 6 mpv. Viral load was calculated as viral RNA copies/mg tissue, expressed as Log10.

	LIVER		*SPLEEN*	
	% RNA positive animals	Log10 viral RNA copies/ mg (positive animals)	% RNA positive animals	Log10 viral RNA copies/ mg (positive animals)
V group (19 surviving rabbits)	5.26%*	3.96	10.32%*	3.54 ± 0.52
C group (10 surviving rabbits)	90.00%	4.13 ± 0.40[a]	100.00%	3.98 ± 0.28[a]
C group (9 dead rabbits)	100.00%	10.76 ± 0.30[b]	100.00%	9.78 ± 0.33[b]

Means with different letters on the same row differ significantly (Student's T-test). * Percentage with significant difference to other rows (Fisher test).

No RHDV-RNA was detected in liver samples from the surviving vaccinated animals, except in 1 animal. In contrast, 95% of the rabbits in the control group showed positive levels of RHDV2-RNA (Table 1). Similarly, 100% of the rabbits in the control group showed positive levels of RHDV2-RNA

in spleen samples, whilst positive levels of RHDV2-RNA were found in 2 vaccinated rabbits (Table 1). Of particular note was the fact that the percentage of positive animals in the vaccinated group was significantly lower than in the control group with both samples (Fisher's test, $p< 0.05$). On the other hand, in post mortem liver and spleen samples from dead rabbits, up to 261 and 246 times higher viral loads respectively were detected than in the survivors (Student's T-test, $p< 0.001$). These findings are in line with results obtained by previous authors (Le Minor *et al.* 2019; Dalton *et al.* 2018).

Differences were observed between groups in the percentage of rabbits in which the virus was detected in faeces. In the non-vaccinated rabbits, the virus was detected in 6 rabbits (out of a total of 10 rabbits sampled) at 1 dpc (60.0%), 5 rabbits at 4 dpc (50.0%) and in 4 rabbit out of 9 at 7 dpc (44.0%), whilst in the vaccinated rabbits, the virus was not detected in any sampled animal at any time. In the faeces of non-vaccinated animals, the highest levels of viral RNA in positive rabbits were detected at 4 dpc when copy numbers in RNA-positive animals were 7.98×10^4 viral RNA copies/mg faeces without significant differences to 2 dpc (6.21×10^4 viral RNA copies/mg faeces; Student's T-test, $p>0.05$) or 7 dpc (6.83×10^4 viral RNA copies/mg faeces; Student's T-test, $p>0.05$).

The findings in the control group are similar to those described by Dalton *et al.* 2018 in different tissues and rectal swabs. Furthermore, this study provides new information about the absence of viral RNA shedding in the faeces of vaccinated rabbits, showing that vaccination prevents RHDV2 spread on farms, as the main dissemination route of RHDV2 (Dalton *et al*. 2018). Similarly, vaccination significantly reduces the RHDV2 viral load in target tissues of vaccinated animals at 6 mpv.

CONCLUSIONS

Vaccination with ERAVAC provides full protection against mortality after experimental challenge and prevents the spread of RHDV in faeces, as well as the persistence of the virus in major target organs, in RHDV2 infected adult rabbits after 6 mpv. This study contributed to describing the effect of the vaccine in RHDV2 transmission, being the main alternative for RHDV2 control on farms.

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