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A novel vaccine candidate against rabbit hemorrhagic disease virus 2 (RHDV2) confers protection in domestic rabbits

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OBJECTIVE
To evaluate efficacy of a novel vaccine against rabbit hemorrhagic disease virus 2 (RHDV2) in domestic rabbits.

ANIMALS
40 New Zealand White rabbits obtained from a commercial breeder.

PROCEDURES
Rabbits were vaccinated and held at the production facility for the duration of the vaccination phase and transferred to Colorado State University for challenge with RHDV2. Rabbits were challenged with oral suspensions containing infectious virus and monitored for clinical disease for up to 10 days. Rabbits that died or were euthanized following infection were necropsied, and livers were evaluated for viral RNA via RT-PCR.

RESULTS
None of the vaccinated animals (0/9) exhibited clinical disease or mortality following infection with RHDV2 while 9/13 (69%) of the control animals succumbed to lethal disease following infection.

CLINICAL RELEVANCE
The novel vaccine described herein provided complete protection against lethal infection following RHDV2 challenge. Outside of emergency use, there are currently no licensed vaccines against RHDV2 on the market in the United States; as such, this vaccine candidate would provide an option for control of this disease now that RHDV2 has become established in North America.

Rabbit hemorrhagic disease virus (RHDV [Lagovirus GI.1]), a calicivirus of the *Lagovirus* genus, is a highly pathogenic RNA virus that infects domestic (*Oryctolagus cuniculus*) and wild European (*Oryctolagus cuniculus*) rabbits.[1,2] The virus was first observed naturally in China in 1984 and was later deliberately introduced in Australia and New Zealand as a method for rabbit population control.[1] RHDV outbreaks in Europe and North America have occurred periodically for the past several decades, and in 2010 the emergence of a distinct genotype, now referred to as RHDV2 (Lagovirus G1.2), was documented in France.[3] Disturbingly, RHDV2 has a wider host range than classic RHDV, encompassing varieties of wild rabbits previously thought to be unaffected by RHDV, including European and other hares (*Lepus spp*) and wild rabbits native to the American continent within the genus *Sylvilagus*.[2–5] In 2016, the first cases of RHDV2 were reported in North America. The virus was initially detected in Quebec and British Columbia, Canada, and it rapidly appeared throughout many states in the continental United States.[2,6]

Both RHDV and RHDV2 are highly pathogenic in domestic rabbits, with a mortality rate ranging from 70 to 100% and death occurring as early as 2 to 3 days postexposure. Young animals (< 8 weeks of age) and wild lagomorphs native to the American continent are resistant to RHDV, but this is not the case with RHDV2, where mortality rates in those groups are similar to those of adult domestic rabbits.[3,7] Both viruses spread extremely quickly from animal to animal via direct contact and/or contact with infectious secretions or via mechanical vectors, and there does not appear to be significant cross-protection between RHDV and RHDV2.[8] Additionally, these viruses are notoriously hardy in the environment and can persist for weeks on various surfaces.[9] In Europe, there are two highly efficacious inactivated commercial vaccines available to

combat RHDV2; an additional recombinant baculovirus vaccine has also been evaluated but is not currently licensed.[10,11] However, these vaccines are not licensed in the United States and are only available on a limited basis. The emergence of RHDV2 in the wild lagomorph population in the United States and the lack of a licensed vaccine put domestic rabbits and wildlife species of concern at an extremely high risk for devastating outbreaks. Thus, it is imperative that an effective vaccine is licensed and available for widescale use in the United States. This study describes an experimental evaluation of a highly efficacious novel vaccine candidate against RHDV2 produced by a US-based organization with the intent to license and manufacture.

Materials and Methods

Animals

Between March and May 2021, New Zealand white rabbits, approximately 3 to 5 weeks of age, from an RHDV2-free commercial facility were assessed for general health and enrolled into randomly assigned blinded treatment groups. Rabbits were not vaccinated against RHDV2 prior to enrollment. They were housed according to site procedures, which was on the doe until weaning and then in groups of approximately 4 in accordance with cage size requirements. Following the vaccination phase, rabbits were transported to Colorado State University (CSU) and group housed by sex for the challenge phase. Rabbits were provided ad libitum water and feed consisting of commercial alfalfa pellets. All procedures at CSU were performed in accordance with University Institutional Animal Care and Use Committee approvals (No. 1161).

Vaccine preparation

The vaccine being tested is an inactivated (killed) baculovirus-derived recombinant subunit vaccine, directed at eliciting an immune response to the immunogenic VP60 protein of RHDV2 (patent pending). The complete sequence of the VP60 protein is shown in **Figure 1**. The product was adjuvanted with aluminum hydroxide to further stimulate the immune response. An adjuvant-matched placebo lacking antigenic proteins was also prepared.

Vaccine administration

On study days 0 and 21, 40 rabbits were vaccinated subcutaneously with a 0.5-mL dose of either the test vaccine (n = 20) or the adjuvant-matched placebo (20). Enrollment to either test group was random and the test product was blinded to all involved in the execution of the study until conclusion.

Virus

Challenge material originated from livers from RHDV2 naturally infected rabbits during the 2020-2021 US outbreak and was supplied by the United States Department of Agriculture (USDA). Challenge material was transferred to the Animal Disease Laboratory at CSU, a large animal Biosafety Level 3 facility (BSL3). Livers were pooled and homogenized in phosphate-buffered saline (PBS) at a 1:1,000 ratio for the starting challenge material.

Challenge

Following the vaccination phase (28 days), a total of 34 rabbits were transported to CSU (6 of the original animals were withdrawn from the study due to confounding illness), 30 of which were group housed in 1 of 2 rooms in the BSL3, sorted by male and female. CSU study participants were blinded to the study groups. The remaining 4 animals were placed in a separate building to serve as uninfected controls. Rabbits were allowed to acclimate for 7 days, during which time they were

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ggtctgcgag aattcatgga aggaaaggct cgcgccgcct cacaagggga aaccgcaggt    60
actgctacta ctgcctctgt gccggggacc actaccgatg gcatggaccc cggagtcgtg   120
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gcggccgcag gtttcga                                                 1817
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Figure 1—Sequence of the VP60 protein expressed by the recombinant baculovirus vector in the vaccine.

Table 1—Treatment groups and survival of rabbits 10 days after experimental challenge with RHDV2. Four control rabbits (placebo control and vaccinate control) were not challenged with RHDV2.

Treatment group	Total enrolled	Surviving	Deceased	Mortality rate
Placebo infected	13	4	9	69%
Vaccinate infected	9	9	0	0%
Placebo control	2	2	0	0%
Vaccinate control	2	2	0	0%

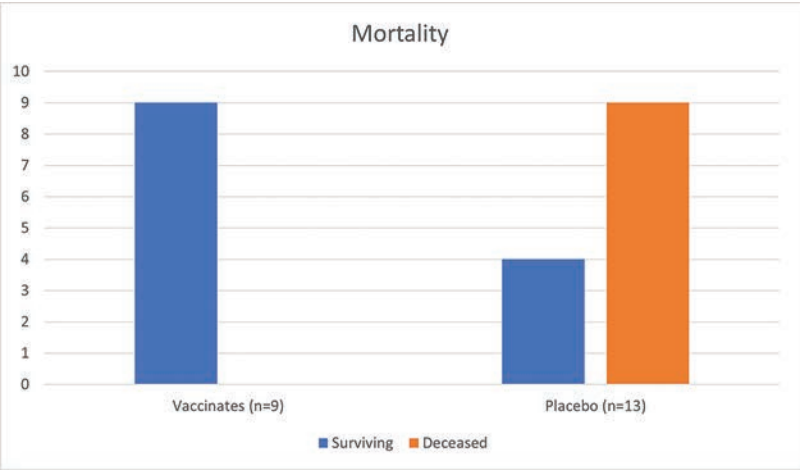

Figure 2—Mortality and survival following challenge in both the vaccinated (n = 9) and placebo (13) test groups of 22 rabbits. Results illustrate significant mortality among the placebo test group while all vaccinated rabbits survived the 10-day postchallenge observation period.

subcutaneously implanted with biothermal microchips, and a baseline blood sample was collected. On day 7 postarrival (day 35 postvaccination), rabbits were challenged orally with 1 mL of a 1:1,000 RHDV2 liver homogenate. Following challenge, rabbits were monitored twice daily for signs of clinical disease and temperatures were recorded daily. Animals who succumbed to infection or were euthanized via pentobarbital overdose due to endpoint criteria (moribund, anorexic > 2 days, dyspneic, hemorrhagic discharge from nose or mouth) were necropsied and livers saved for by real-time reverse transcriptase (RT-PCR) analysis. All other rabbits were euthanized on day 10 postinfection (45 days postvaccination), and livers were harvested for RT-PCR analysis.

RT-PCR analysis

Challenge material and livers from all rabbits were tested for the presence of RHDV2 RNA RT-PCR as previously described.[12] Briefly, livers were prepared for extraction by homogenizing in lysis buffer using Qiagen RNeasy extraction kits per manufacturer's instructions (Qiagen), and RT-PCR was performed using TaqMan Fast Virus 1-step Mastermix kit (Thermo Fisher Scientific).

Statistics

A 2 X 2 contingency table was executed using a Fisher exact test, with a 2-tailed *P* value on the mortality across the 2 treatment groups.

Results

Out of the 34 animals initially transported to CSU, several animals (n = 8) were excluded from analysis due to illegible ear tattoos, which precluded being able to properly identify their vaccination status. The remaining 26 animals belonged to either the vaccinated group (n = 11) or the placebo group (15). Two animals from each group were kept as uninfected controls in a separate building, leaving 9 animals in the vaccinated + infected group and 13 animals in the placebo + infected group **(Table 1)**. Of those animals, 9/13 (69%) in the placebo group died or were euthanized due to endpoint criteria following RHDV2 infection while 0/9 of the vaccinates died or were euthanized prior to the study endpoint (*P* value of .0017; **Figure 2**). The mortality rate of the placebo group is consistent with other studies and with preliminary experimental infections (data not shown), and is indicative of a lack of immunity in this group, while the complete protection from death in the vaccinated animals indicates a highly successful response to vaccination. Livers from all animals were evaluated via RT-PCR and the cycle threshold (CT) values in rabbits that succumbed to disease were considerably lower compared to survivors **(Figure 3)**, with the average CT value in diseased animals of 16.1 and the average in animals that survived a challenge of 27.6 **(Table 2)**. No viral RNA was detected in the livers of control animals.

Because animals were group housed, clinical monitoring was based on body temperature and twice daily temperament observations and did not include anorexia or fecal output. According to the *Handbook of Clinical Signs in Rodents and Rabbits* (1st ed), the Charles River, New Zealand white rabbit's normal temperature range is from 100.4 to 104 °F. The four control rabbit temperatures ranged from 102.4 to 104.4 °F in this study, so for this RHVD2 challenge study, we considered any temperature > 104.4 °F to be febrile. All but 1 animal that succumbed to disease exhibited fever > 105 °F in the day(s) preceding death. Interestingly, 3/9 (Nos. 27, 35, and 40) vaccinates also developed a fever > 104.4 °F for at least 1 day

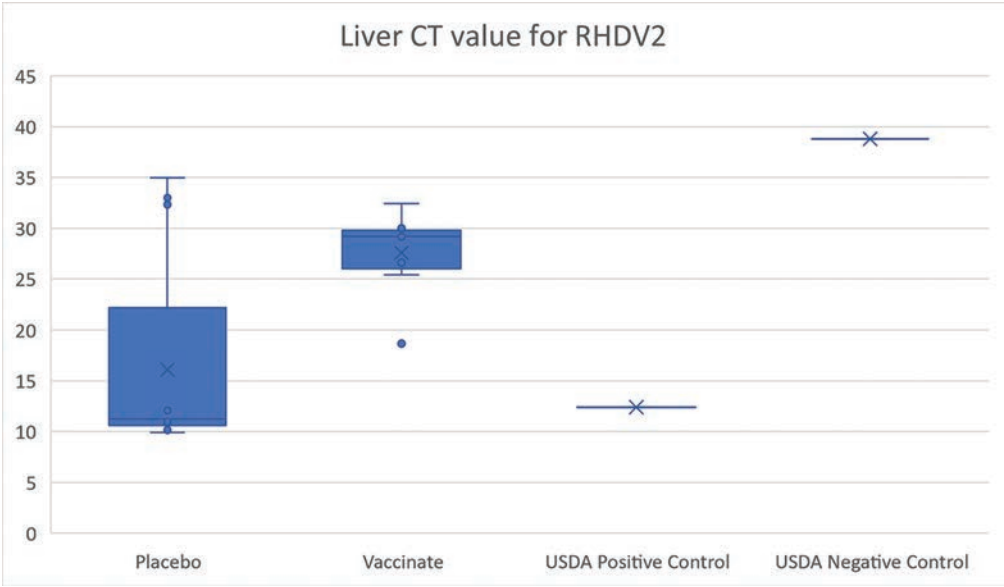

Figure 3—Graphical representation of the RT-PCR results across both vaccinated and placebo treatment groups of 22 rabbits. For comparison, RHDV2-positive (cycle threshold [CT] = 12.4) and -negative (CT = 38.8) control livers were provided by the USDA. All livers tested following direct challenge with RHDV2 did yield PCR CT counts less than the negative control provided by the USDA. However, vaccinated animals do appear to have higher CT counts (indicative of lower viral loads) in comparison to the nonvaccinated animals.

Table 2—Individual rabbit RT-PCR results from liver samples following experimental challenge with RHDV2 (n = 22).

Liver PCR Cycle Threshold (CT) Values

Placebos			Vaccinates		Assay Controls	
Rabbit ID	**CT**	**Days postinefection (died/euthanized)**	**Rabbit ID**	**CT**	**Sample**	**CT**
3	10.956	3 (died)	12	25.422		
5	11.241	3 (died)	15	26.943	USDA positive control	12.416
13	10.57	3 (died)	20	29.353	Nontemplate control (water)	Undetermined
16	11.113	3 (died)	22	26.602	1:1,000 Challenge material	20.598
21	11.278	3 (died)	25	29.989		
24	10.169	3 (died)	26	29.159		
28	10.624	3 (died)	27	29.597		
30	9.93	10 (euthanized)	35	18.669		
31	12.081	6 (died)	40	32.412		
33	32.301	10 (euthanized)				
34	11.363	8 (died)				
38	34.957	10 (euthanized)				
39	32.996	10 (euthanized)				
	Group average			Group average		
	16.1			27.6		

over the course of the study, but all of these animals' temperatures normalized prior to the study endpoint, and no additional clinical signs were observed. In general, sudden death occurred without prior observable clinical signs; rarely, presentation was laterally recumbent/moribund during clinical observation, and these animals were immediately euthanized. Animals that developed fevers but recovered did not display any other clinical signs of disease.

Discussion

RHDV2 is a disease of considerable concern for lagomorphs. Since its introduction in 2016, the virus has spread across much of the United States and into Canada and at the time of this publication has been detected in wild rabbits from 11 states and domestic rabbits from 19 states (https://wildlifehealth.org/rabbit-hemorrhagic-disease-virus/). Biosecurity practices can be effective at preventing

infection in large breeding facilities, but caliciviruses are extremely hardy in the environment, and it has been demonstrated that RHDV, and likely RHDV2, can be transmitted via mechanical insect vectors and that both strains can spread via infectious fecal material.[13,14] Viral RNA can be detected in animals that survive infection for up to 3 months, which could represent a continual reservoir for infection of naïve rabbits.[15] Furthermore, cottontails (*Sylvilagus spp*), which are susceptible to disease but do not always die after infection, are prolific across the country and can be in close contact with domestic rabbits in certain situations, particularly those that live as pets in backyards.[4] Clearly, the best means of preventing mortality is vaccination, and the lack of a readily available vaccine in the United States is concerning for rabbitries, pet owners, and wildlife managers. Thus, the vaccine described herein is timely and will be useful to prevent further infections of rabbits in the United States. In this study, vaccination was 100% effective at preventing mortality, while 70% of unvaccinated animals died following exposure to the virus. Three vaccinated animals developed fevers for 1 to 3 days following infection, similar to previous vaccine trials, which is possibly due to response to infection, considering that vaccination does not prevent infection but rather prevents significant disease and mortality.[10] While vaccinated-infected animals still retained viral RNA in tissues following infection, prevention of clinical disease and mortality was the primary goal of the study and the most important outcome for vaccination efforts against RHDV2.

The time to death for most animals infected with either RHDV or RHDV2 is generally 48 to 72 hours postexposure, and sick animals are capable of shedding large quantities of virus into the environment and can serve as a source of infection even after death.[5] Therefore, an introduction of even 1 infected animal into a herd can lead to massive and unavoidable death loss unless the herd is immune. Because of the rapidity of spread and rapid disease pathogenesis, some producers may opt for depopulation, which is clearly undesirable and can be avoided by widespread vaccination efforts.

There are several limitations to this study, primarily that vaccination does not prevent infection, and vaccinated animals may still be able to shed infectious virus, so the best practice available is widespread vaccination of susceptible populations. Future studies are needed to evaluate the ability of vaccinated-infected animals to shed sufficient virus to infect unvaccinated animals. Ideally, vaccination efforts would include all domestic rabbits, including pets and farmed animals, as well as species of concern among wild lagomorphs (i.e. various pika [*Ochotona spp*] and hares [*Lepus spp*]). While the goal of this study was not to evaluate the vaccine in wildlife, there is clearly a conservation need to do so. Additional limitations include small sample size, particularly as some animals were excluded from the study due to lack of clear tattoo identification; this can easily be rectified in future studies by including additional forms of identification (i.e. microchips) at the onset of the study. Additional studies to address both the safety profile of the vaccine and duration of immunity are also needed. Finally, this study only evaluated vaccination in very young animals (3 to 5 weeks old); it is possible that there could be age-related differences in the immune response to the vaccine, and as such, future vaccine trials will need to address juvenile versus adult vaccine efficacy.

Because lagoviruses cannnot be grown in cell culture, traditional inactivated vaccines must be derived from the livers of RHDV2-infected animals, which requires infecting and euthanizing rabbits for this purpose. Recombinant vaccines for RHDV2 such as the one described herein and others provide an advantage over inactivated vaccines in that there is no need to use live animals or infectious virus to derive the vaccine, thus saving lives and eliminating the possibility of reversion to virulence.[10]

RHDV2 is a clear example of an emerging infectious disease that, once established, will not be easy to eradicate. In the United States and other parts of the world, this disease has become endemic in a very short period of time, and thus, control strategies implemented across boundaries are needed.[16] Vaccines such as the one evaluated herein are necessary for preventing the spread of disease and mass mortality events in rabbit populations.

Acknowledgments

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