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RHD-Boost: Import and evaluate new rabbit haemorrhagic disease virus (RHDV) variants to strengthen rabbit biocontrol

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PESTSMART

RHD-Boost

Import and evaluate new rabbit haemorrhagic disease virus (RHDV) variants to strengthen rabbit biocontrol

Report to the Vertebrate Pests Committee

Endorsed by the Invasive Animals CRC
Rabbit Biocontrol Scientific Committee

Invasive Animals CRC

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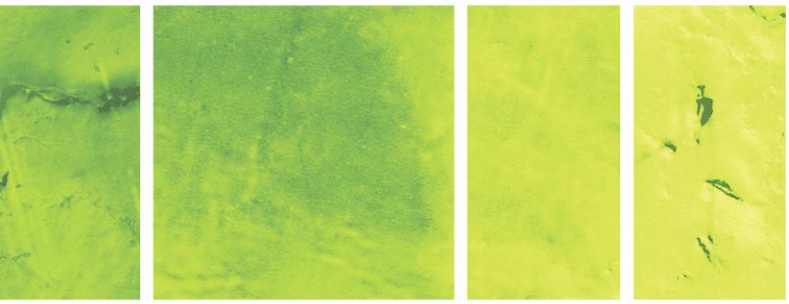

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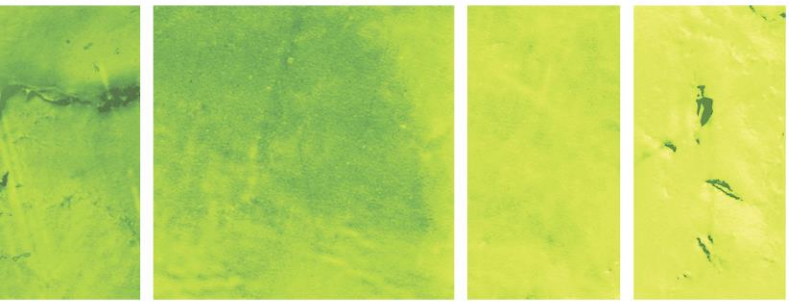


Executive Summary

The **RHD Boost** project sought to identify new RHDV variants with superior lethality to rabbits with partial protection from endemic Australian Rabbit Calicivirus (RCV-A1) and immunity and/or genetic resistance to infection with existing Australian Czech 351 derived RHDV variants. Any new RHDV strain with greater lethality in rabbits with RCV-A1 is predicted to result in benefits in cooler-wetter regions where RCV-A1 is prevalent, and coincides with higher agricultural and environmental productivity.

The project is a strategic response to the apparent rising genetic resistance to the RHDV Czech 351 RHDV variant, and its limited effectiveness in temperate regions due to the prevalence of the non-pathogenic endemic RCV-A1, which can provide transient protection to lethal infection with RHDV. In Europe, new RHDVa variants are reportedly out-competing the original classical RHDV variants in the field and strongly suppressing wild rabbit populations in cooler, wetter regions.

RHD Boost was a government-industry partnership funded by the Australian Government Caring for Our Country Program, NSW Department of Primary Industries, CSIRO, Australian Wool Innovation, Meat and Livestock Australia and Rabbit Free Australia and managed by the Invasive Animals CRC.

Candidate RHDV variants selected for testing in Australia as part of this trial were chosen on the following criteria:

1. Increased genetic and antigenic variation from the Czech v351 RHDV variant. RHDVa variants were considered preferable to classical RHDV variants. The Czech v351 variant that has been released in Australia belongs to the classical group. Therefore selection of RHDVa variants should increase the likelihood of significant genetic and antigenic variability.
2. Isolates that were antigenically distinct or exhibited different biological traits from the Czech v351 variant.
3. Variants that apparently displaced others in Europe or North America as evidence of potential competitive advantage over the Czech v351 variant.
4. Variants that may overcome the partial temporal protection of the endemic benign calicivirus RCV-A1.

Thirty-eight genetically and antigenically different RHDV variants and one RHDV-like virus (RHDV2) were imported and evaluated as part of this work. These variants represented all the recognised genetic lineages. Six of these were selected for further testing, along with one highly virulent Australian field variant. The key findings and recommendations that have come out of the research undertaken as part of the RHD-Boost project are as follows;

Key Findings:

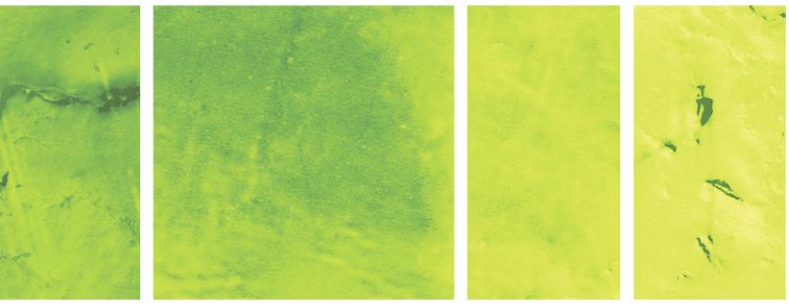
- Two variants from South Korea (K5 and K9) demonstrated advantages over the Czech variant currently available in Australia and warrant consideration as biocontrol agents in Australia. In particular, the K5 variant has been shown to overcome the partial protection offered by the endemic benign calicivirus RCV-A1.
- None of the variants that were able to overcome immunity to the Czech RHDV. The

RHDV2 virus variant X15 did demonstrate a moderate ability to overcome antibodies to the Czech RHDV. While this virus has similar pathogenic traits to RHDV, it is a new virus type. However, RHDV2 has been implicated as the cause of several outbreaks of acute hepatitis in rabbits and Cape hares (*Lepus capensis mediterraneus*) in Sardinia. This is the first report of a lagovirus causing fatal hepatitis in both rabbits and hares (Puggioni *et al*. 2013). Despite the host range including only lagomorphs, this broader host specificity may make RHDV2 variants less attractive as a biological control agent due to concerns about the potential of the virus to infect non-target species.

- Both the Czech RHDV and the K5 virus variant are shed by kittens and can be transmitted to bystander rabbits, however the importance of this in relation to the effectiveness of RHDV requires further investigation under field conditions. An enhanced transmissibility of K5 was not observed.
- Modelling studies suggest that there may be difficulty in establishing new RHDV variants, although short-term success in specific localities seems likely if new variants are strategically released ahead of expected outbreaks of field-variant.

Key Recommendations:

- The K5 (South Korea 08Q712-1) variant has demonstrated advantages over the Czech v351 variant currently available in Australia. The ability to infect rabbits previously infected with RCV-A1 is likely to make this variant more effective than Czech-derived field variants of RHDV currently circulating in Australian rabbits. As such, this variant appears suitable as a biological control agentand warrants field investigation. We therefore recommend that this variant should be selected as a candidate for rabbit biological control in Australia.
- The use of K5 should be in accordance with a national RHDV-K5 release and performance monitoring plan.



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Introduction

Wild European rabbits (*Oryctolagus cuniculus*) were successfully released in 1859 and spread quickly through the Australian environment. Within 62 years the rabbit population had an estimated distribution of 69% or 5.3 million km^2 of the Australian continent. Rabbits are now found in all states and territories (Figure 1). Rabbits can utilise a wide range of habitats including Australia's arid interior.

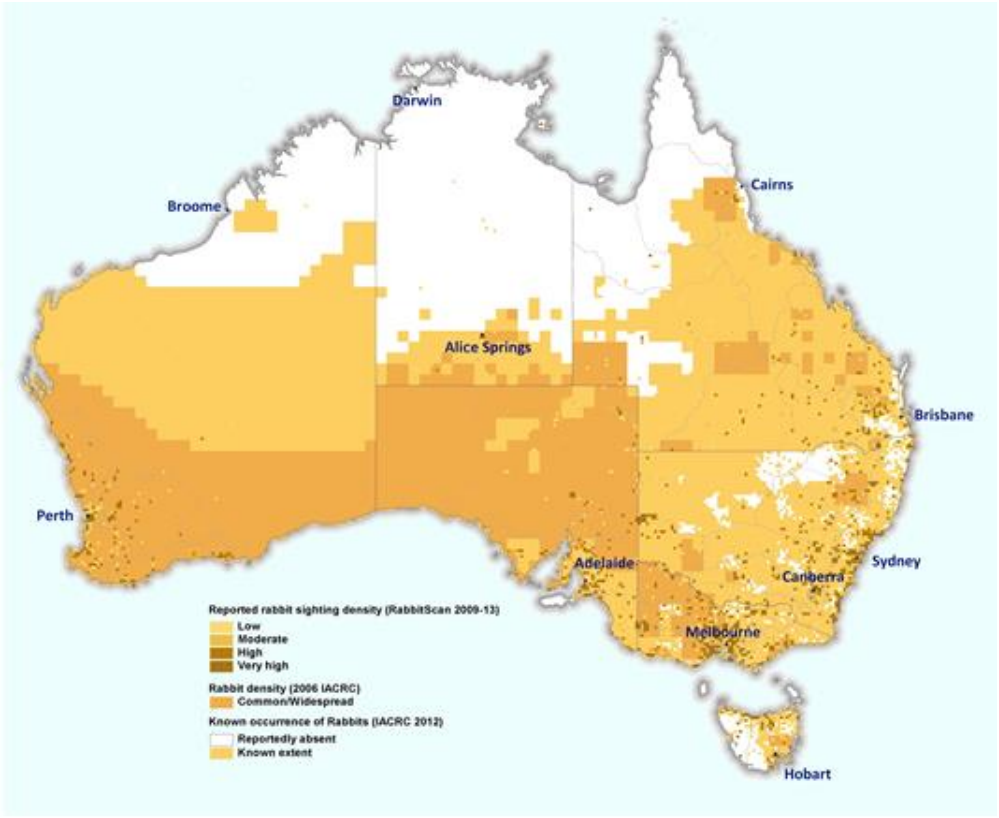
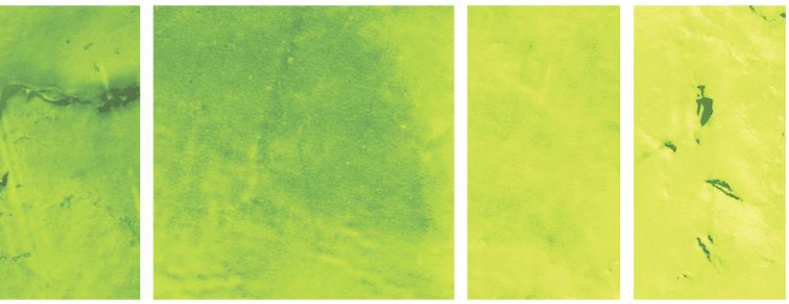


Figure 1: Rabbit occurrence, abundance and distribution across Australia (Source data: Invasive Animals Cooperative Research Centre, RabbitScan, and National Land & Water Resources Audit and Invasive Animals Cooperative Research Centre 2008).

European rabbits are the most economically and environmentally important vertebrate pest species in Australia. It has been estimated that damage to crops and pastures exceeds $200 million annually, with a further $6 million spent on control annually (McLeod 2004, Gong et al. 2012). In addition to direct economic loss, they cause extensive damage to the environment by destroying native vegetation and competing with and displacing native animals from their ecosystems.

Rabbits compete with native animals and domestic stock for food resources, and overgraze native plants. Their grazing and burrowing activities destabilise soil systems and undermine geomorphic processes; contribute to erosion and the undermining of soil integrity, as well as



altering the structure and composition of vegetation communities (Department of the Environment Water Heritage and the Arts 2008). Rabbits are also highly fecund and their high population size helps support populations of other introduced species such as foxes and cats (Bowen and Read 1998).

Rabbits impact negatively on many native species: 35 species of animal (19 birds, 13 mammals, 2 reptiles and 1 insect) and 121 species of plant are directly threatened. Of these plants and animals, 69 are vulnerable to extinction, 78 species are in danger of extinction, and 9 species are in critical danger of extinction (Department of the Environment Water Heritage and the Arts 2008). Many more animals, plants and vegetation communities are indirectly affected by rabbits and their activities. Competition with, and land degradation caused by, rabbits are listed as key threatening processes under Schedule 3 of the Commonwealth *Environment Protection and Biodiversity Conservation Act 1999*.

Control measures such as poisoning, fencing and warren destruction have helped to reduce rabbit numbers in some regions, but biological control has had the greatest impact. Myxoma virus (MV) was introduced to Australia in 1950. The virus initially killed 99% of experimentally infected rabbits and reduced numbers by over 90% in the Riverina District where it was first released (Myers 1962). In the following five years, rabbit numbers were reduced by approximately 83% across Australia, however, a reduction in virus virulence and the development of genetic resistance in rabbits led to a gradual increase in the rabbit population over the ensuing 30 years.

RHD in Australia

Rabbit haemorrhagic disease virus (RHDV) (Czech v351 variant) was first brought to Australia in 1991 to be tested as a potential biological control for wild rabbits. RHDV is a lagovirus (Family Caliciviridae) that causes a mostly fatal infective disease (RHD) in European rabbits. RHDV was first described in domestic rabbits in China in 1984. In 1995 RHDV escaped quarantine into the Australian rabbit population and was subsequently released in a coordinated program in 1996 (Mutze *et al*. 2010a). Its effects on rabbits were variable with the greatest impacts in arid and semi-arid inland areas, reducing populations by 80-95% (Bowen & Read 1998, Henzell *et al*. 2002, Mutze *et al*. 2008). RHDV was least effective in coastal areas, in cool moist areas, and during summer in areas of summer rainfall (Henzell *et al*. 2002).

The patchy success of RHDV has been, for the most part, attributed to the presence of the benign calicivirus rabbit calicivirus Australia 1 (RCV-A1) which has long been suspected of reducing the effectiveness of RHDV in Australia (Figure 2, Cooke *et al*. 2000, Nagesha *et al*. 2000, Cooke *et al*. 2002, Robinson *et al*. 2002). In 2008 RCV-A1 (Rabbit Calicivirus – Australia 1) was identified and characterised (Strive *et al*. 2009). RCV-A1 causes an entirely non-pathogenic infection of the upper intestinal tract and offers partial cross-protection to RHDV (Strive *et al*. 2010).

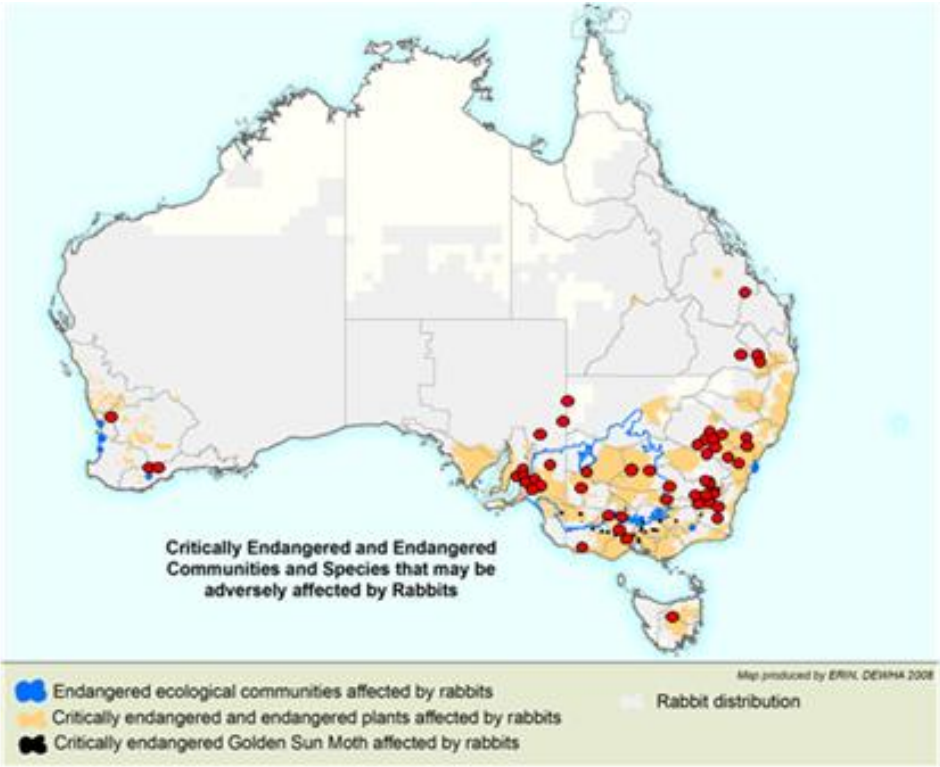
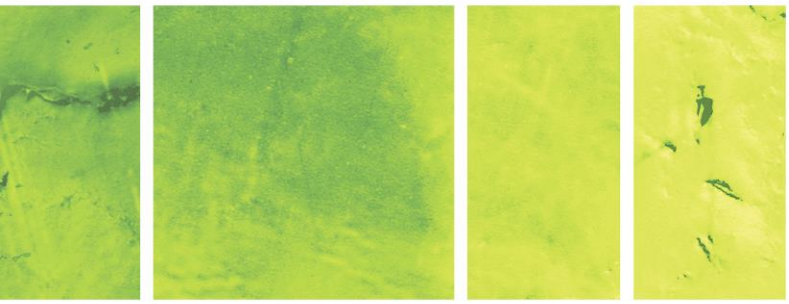


Figure 2: Distribution of the benign endemic calicivirus RCV-A1 in Australia. Red dots indicate current and historical sites that have tested positive for RCV-A1. (Commonwealth of Australia 2008, RCV-A1 data from Liu et al., in preparation).

RHDV appeared to keep rabbit numbers greatly reduced for approximately a decade following its establishment. However, in more recent years the wild rabbit population in Australia has increased (Mutze *et al*. 2010a,b). Further to this there is evidence that some populations of rabbit are developing genetic resistance to RHDV. The most advanced work to date has been carried out by Nystrom *et al*. (2011) who have identified the role that histo-blood group antigens (HBGAs) play in RHDV binding in the rabbit. Rabbits without the correct HBGA ligands were resistant to infection with RHDV at low doses. The data from Nystrom *et al*. (2011) identify the population at Hattah-Kulkyne National Park in northwest Victoria as a site with weak-binding phenotypes and therefore a possible genetically resistant population. Recent sampling of the Hattah population (Cox unpublished data) revealed that RHDV antibody prevalence has been consistently high at Hattah (73–95%). RCV-A1 prevalence was low (0–33%) suggesting that protection by RCV-A1 was not the major factor in the high rates of RHDV seroconversion, lending support to the findings of Nystrom *et al*. (2011).

The work described in this report is a response to the waning effectiveness of RHDV-mediated biocontrol, due to apparent rising genetic resistance of rabbits to the Czech variant or the protective effects of RCV-A1. The aim of the RHD-Boost research project was to identify overseas RHDV variants that may overcome protection from RCV-A1 and/or genetic and acquired resistance, and to assess if the seemingly superior new RHDV variants that are emerging world-wide can complement the existing Australian field variants and improve rabbit biocontrol success.



Evaluation of new variants of RHDV

The key activities reported here include the selection of overseas RHDV variants, testing to ensure freedom from other adventitious disease agents, production of virus stock, screening of virus variants for ability to overcome immunity to RCV-A1, the screening of wild rabbits for ability to overcome immunity to RHDV and whether the new candidate variant/s have any competitive advantage over the Czech variant.

Selection of variants

Until the late 1990s, there was only one known RHDV genotype. Further research has identified that RHDV can be divided into six genogroups (G1-G6) and other non-pathogenic or benign caliciviruses (RCVs). The G1-5 genogroups consist of the more classical RHDV, while the G6 group consists of the antigenic variant RHDVa, a subtype of the RHDV wild-type (Abrantes *et al*. 2012) first identified in 1998 (Capucci *et al*. 1998). G6 variants have been discovered in Europe, the USA and South East Asia and appear to be replacing the established G1-5 variants (like the Czech v351 variant, hereafter referred to as 'the Czech variant', which belongs to the G1 genogroup) in Eurasia, suggesting that G6 variants in general may have a competitive advantage over G1-5 variants (McIntosh *et al*. 2007). In 2012/13 a group of RHDV viruses that was antigenically and genetically distinct from all genogroups of RHDV was described, and designated RHDV2. RHDV2 is a new virus and has been reported to overcome antibodies to RHDV (Puggioni *et al*. 2013).

Candidate RHDV variants selected for testing in Australia (Table 2) were chosen on the following basis:

- G6 variants (RHDVa) were thought preferable to G1-5 variants (like the Czech variant) because they are likely to have greater genetic and antigenic variability.
- Isolates that were antigenically distinct from the Czech variant were preferentially investigated.
- Variants that apparently displaced others in Europe or North America also offered a potential competitive advantage over the Czech variant.

Table 1: Identification of the candidate RHDV variants selected for testing in Australia

Variant Identifier	Number of samples	Group	Origin
K	10	G1-6	South Korea
F	3	G6 & unknown	France
E	180*	G1-6	Spain
X	22	G6 & RHDV2	Spain/Portugal
C	2	G6	China
Tur09	1	G1	Turretfield Research site, Australia
RCV-A1	1	Benign	CSIRO, Australia

*of the 180 samples supplied only 25 isolates had suitable quantities of virus and were further investigated.

Methods

The work described here was undertaken at the Elizabeth McArthur Agricultural Institute (EMAI), New South Wales by both NSW Primary **Industries** and CSIRO researchers.

Variant characterisation

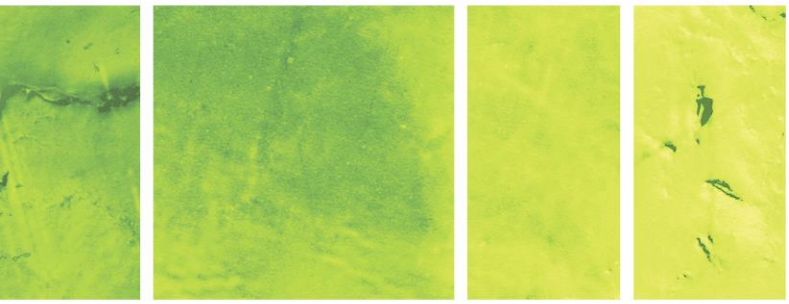
All of the isolates from South Korea (sample prefix 'K'), France (sample prefix 'F') and Spain (sample prefix 'E'), which contained sufficient antigen, were tested at the World Reference Laboratory to determine whether they belonged to Group I or Group II. RHDV2 isolates (sample prefix 'X') were from Spain and Portugal. Chinese isolates (sample prefix 'C') were not antigenically characterised.

Preparation of virus stocks

Pre-master and master stocks were prepared from the original Czech variant, the Australian variant (Tur09), the benign variant (RCV-A1) and those international variants that were sufficiently distinct from the Czech variant (after variant characterisation) (Table 3). Pre-master and master stocks were produced in 14 week old specific pathogen free (SPF) New Zealand White rabbits (*O. cuniculus*).

Table 2: Summary of viruses from which pre-master and master stocks of viruses were used.

Name	Origin	Name	Origin
K1	South Korea	Tur09	Australia
K2	South Korea	X1	Spain
K3	South Korea	X4	Portugal
K5	South Korea	X11	Spain
K6	South Korea	X15	Spain
K7	South Korea	X19	Spain
K8	South Korea	X20	Spain
K9	South Korea	C1	China
K10	South Korea	C2	China
E9	Spain	Czech	Czechoslovakia
F1	France	RCA-A1	Australia
F2	France		
F3	France		



Adventitious agent testing

Australia maintains a cautious approach to the management of quarantine risk. This is particularly important in the case of live virus preparations because they are not subjected to microbiologically lethal treatment during production. The Australian Government Department of Agriculture considers that imported live vaccines present inherently high quarantine risks due to the direct exposure of live animals to these vaccines. A decision to permit imports depends upon a detailed and rigorous technical assessment of the raw materials, their processing and the testing of the final product. There is a requirement for live imported viruses to be strictly controlled and products must be tested for pathogens of quarantine concern using sensitive methods. The testing required is determined by the Australian Government Department of Agriculture.

The Australian Government Department of Agriculture Biological Imports Program has recently assessed what testing is specifically required for imported RHDV variants under the guidelines outlined in the Australian Government Department of Agriculture documents 'Australian Quarantine Policy and Requirements for the Importation of Live and Novel Veterinary Bulk and Finished Vaccines, November 1999' and 'Review of Published Tests to Detect Pathogens in Veterinary Vaccines Intended for Importation into Australia July 2011.' Testing for adventitious agents on the selected variant has commenced.

Preparation of test rabbits

Cross-bred 7–8 week old rabbits of mixed sexes (obtained from a commercial rabbit producer), and offspring of wild rabbits from Bulloo Downs in southwest Queensland which survived exposure to RHDV (supplied by DAFF Qld) at between 4 and 20 months of age, were used to develop the test groups. To produce rabbits with RHDV antibodies, all clean (seronegative) commercial rabbits were vaccinated with one quarter (0.25ml) of the recommended dose of Cylap® vaccine. Any rabbits that did not develop a detectable antibody response to RHDV within four weeks were given a second dose of 0.25ml Cylap® and retested three weeks later to ensure all rabbits had antibodies against RHDV. To produce rabbits with RCV-A1 antibodies, clean (seronegative) rabbits were orally dosed with 1ml of a suspension containing RCV-A1. Rabbits were tested after four to seven weeks to confirm that each rabbit had antibodies to RCV-A1. All wild rabbits were seronegative when tested, and were at least 12 weeks of age at the time of RHDV challenge.

Titration in test rabbits

Where possible, an infectious dose (ID_{50}) was calculated for domestic rabbits with antibodies against RHDV, domestic rabbits with antibodies against RCV-A1 and in wild rabbits. Wild rabbits and domestic rabbits with antibodies against RCV-A1 were exposed to each virus variant at four concentrations: 100, 10, 1 and 0.1 ID_{50}/ml for each virus. Rabbits with antibodies against RHDV were exposed to each virus variant at three concentrations: 10^3, 10^2 and 10^1 ID_{50}/ml for each virus. The original Czech variant was used as a control and to correlate results between different trials.

Competitive advantage trials

Trials were undertaken to determine whether a G6 variant was more competitive than the G1 Czech variant. The likelihood of recombination between two different variants in a mixed infection was also investigated. Rabbit kittens (4–5 weeks old) were used so that investigations into whether kittens are potentially more resistant to infection and/or disease with the selected Group II variant could be undertaken. Rabbit kittens are believed to play a key role in RHDV epidemiology, as they are resistant to lethal RHDV infection, but can get infected and shed virus into the environment. Kittens are more resistant to RHD the younger they are and it has been speculated that they may carry the virus without signs of disease for prolonged periods (Ferreira *et al*. 2004). In this way, rabbit kittens may significantly influence RHDV field epidemiology and attempts to control rabbit numbers. Unfortunately, our understanding about the role of rabbit kittens in RHDV epidemiology is limited and little quantitative data exist in regard to the extent RHDV replicates in, and is shed from, very young rabbits. The Korean variant K5 was selected for comparison. The recombination aspect of the project was investigated using the Czech variant and the non-pathogenic RCV-A1. At the time this work had to commence, the imported variants had not yet arrived in Australia, so this aspect had to be carried out on two endemic calicivirus variants.

Comparative growth curves

Two separate comparative growth trials were carried out using kittens of two litters that were 4.5 weeks of age. In the first trial kittens were infected with a moderate dose (700-1800 ID_{50}). In the second trial, kittens were infected with a very high dose (6x more Czech variant and 26x more K5 compared to the low dose infections). Virus growth and shedding was monitored by collecting daily blood and rectal swab samples for up to five days post infection when kittens were euthanased and tissues and bile were collected.

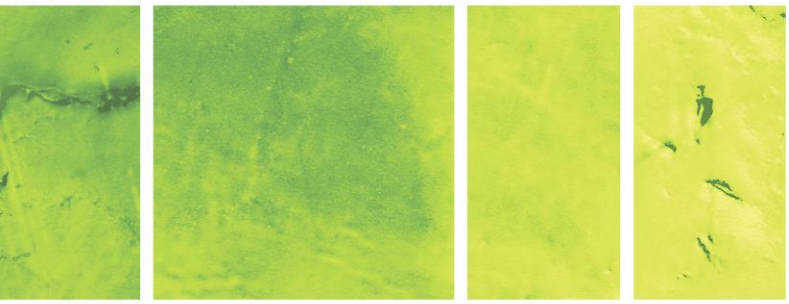
Transmission trials

Kittens of three litters were used to carry out transmission experiments so that the experiments could be replicated three times. Four infected kittens were used per experiment (two kittens infected with the Czech variant and two with K5). Infected kittens were housed separately for one day before they were placed into a group cage with four uninfected littermates. At Day 2, two of the infected kittens were removed, euthanased and tissues were collected. The remaining animals were euthanased at Day 5 post infection. Quantification of the virus loads was carried out using a quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) assay that detects both variants. Analysis of the bystander rabbits was carried out using specific qRT-PCT tests for either the Czech variant or K5.

Sample analysis

Real-time PCR was used to determine the relative quantities of viral RNA in samples. The assay was performed as described by Strive *et al*. (2010) with the addition of an oligonucleotide probe developed at EMAI.

Enzyme-linked immunosorbent assays (ELISAs) were used to detect the presence of virus



antigens in tissues or specific antibodies in rabbit sera. Three ELISAs were used throughout this trial. The RHDV antigen capture ELISA is an assay that is designed to capture RHDV proteins using hyperimmune anti-serum. It was used to detect virus in rabbit liver samples. The RHDV antibody ELISA (as previously described Capucci *et al*. 1991 and Capucci *et al*. 1997) was used to detect antibodies specific for RHDV. Finally the RCV-A1 antibody ELISA (as previously described Liu *et al*. 2012) was used to detect antibodies specific for RCV-A1.

Results

Variant Characterisation

Of the South Korean variants, K1 was the only G1-5 variant. All other variants belonged to genogroup G6I. K5 and K9 showed the greatest antigenic variation when compared to the control G6 viruses.

Of the 25 Spanish samples selected for further investigation, 18 had sufficient antigen to perform further screening. Isolates E5–E7, E9–E17, E20, E23 and E33 belonged to the G1-5 genogroups. Variant E9 appeared to show the greatest variation. Isolates E25, E27 and E32 were clearly G6 genogroup variants.

Initial screening of the French variants indicated that they were antigenically different to both the G1-5 and G6 variants. Unfortunately, the isolates that were forwarded to EMAI were mixtures of this antigenically different variant and a G6 variant. Further evaluation of the French variants was not undertaken.

The isolates X4 and X11 were shown antigenically to belong to the G6 genogroup, while X15 belonged to the RHDV2 group of viruses. Isolates C1 and C2 were shown to antigenically belong to the G6 genogroup. Tur09 was shown to belong to the G1-5 genogroup (specifically G1, but interestingly, it had changes to a key epitope that made it different from other G1 variants).

Preparation of virus stocks

RHDV variants K5, K9, E9, Tur09, C1, the RHDV2 variant X15 and the Czech variant were chosen as candidate viruses for further experimental work based on their antigenic and genetic characteristics. Pre-master and master stocks of these variants were produced. The master stocks for these viruses were titrated in domestic rabbits to determine the infectious dose in each stock (Table 4). Real time PCR testing on the titrated stocks was used to calculate the quantity of viral RNA contained in 1 $ID_{50,}$ as determined by the Ct values from the qRT-PCR. A lower Ct value indicates that there is more viral RNA in the sample. Interestingly the number of copies of viral RNA found 1 RID_{50} can vary from approximately 60 to 14000. It is presumed that this difference is due to variable numbers of non-infectious virus particles present in each master stock preparation.

Table 3: Titrations of Master Stocks of RHDV.

Variant	Concentration (ID_{50}/ml)	Ct value at 1 RID_{50}/ml	Inferred number of virus particles equivalent to 1 RID_{50}
K5	84140	33.1	75
K9	17783	25.5	14000
E9	8050	27.0	5100
X15	17782794	NA*	NA*
C1	23865898	35.8	10
Tur09	1632172	33.4	61
Czech variant	34717	30.9	340

*Variant X15 could not be measured with qRT-PCR, as the genetic sequences were too different to be recognised by the oligonucleotides used in this assay. However, a semi-quantitative analysis using a standard PCR assay that detects all lagomorph caliciviruses (Strive et al., 2009) confirmed the presence of high levels of a lagovirus in this sample.

Titration in test rabbits

Titration in rabbits with RHDV antibodies

Commercial rabbits with antibodies to RHDV were infected with 10, 100 or 1000 ID_{50} of each virus orally. With the exception of the X15 virus, none of the viruses caused infection in these rabbits. X15 caused the death of two rabbits in each dilution group. The number that succumbed to the infection did not appear to be dose related. Although a small number of individual rabbits became infected with the E9 and C1 variants, this trial indicated that rabbits with antibodies against RHDV were mostly protected against a dose of at least 1000 ID_{50} from RHDV variants K5, K9, E9, Tur09 and Czech variant when given orally. With the exception of X15 no conclusion regarding differences between viruses in the ability to overcome RHDV immunity could be made from these results. The results for X15, though not definitive, show some indications that it can at least partially overcome immunity to RHDV.

Titration in rabbits with RCV-A1 antibodies

Commercial rabbits with antibodies to RCV-A1 were infected with 0.1, 1, 10 or 100 ID_{50} of each variant orally. It was found that it required almost 100 times the oral dose of the Tur09 and X15 variants, 40 times the oral dose of the Czech variant, 18 times the oral dose of E9, and 2 times the oral dose of K5 in rabbits previously infected with RCV-A1 to achieve the same infection rate as that observed in naïve rabbits (Table 5). This trial demonstrated that previous infection with RCV-A1 did provide some degree of protection against a subsequent infection with the Czech variant and the E9 variant but not against the K5 variant.

Table 4: The ID50 required to infect 50% of rabbits with RCV-A1 antibodies for the E9, K5, K9, C1, X15, Tur09 and Czech variant viruses.

Variant	Dose (ID_{50}) required to infect 50% of rabbits with RCV-A1 antibodies
Czech variant	40
E9	18
K5	2
K9	13.5
C1	55
X15	100
Tur09	100

Titration in wild rabbits

In seronegative wild rabbits, about 18 ID_{50} of the Czech variant were needed to infect 50% of the rabbits, while 4 ID_{50} were needed of the Tur09 variant, and about 0.6 ID_{50} were needed for the K5 variant (Figure 3). These data indicate that 30 times more Czech variant virus than K5 virus is required to infect these wild rabbits.

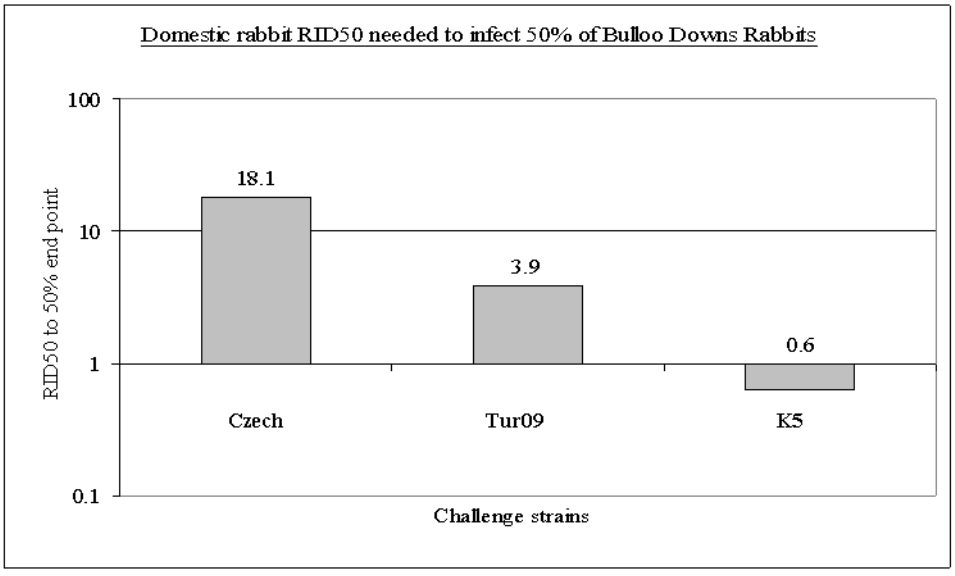


Figure 3: ID_{50} (as calculated in domestic rabbits) required to infect rabbits from Bulloo Downs for three RHDV variants.

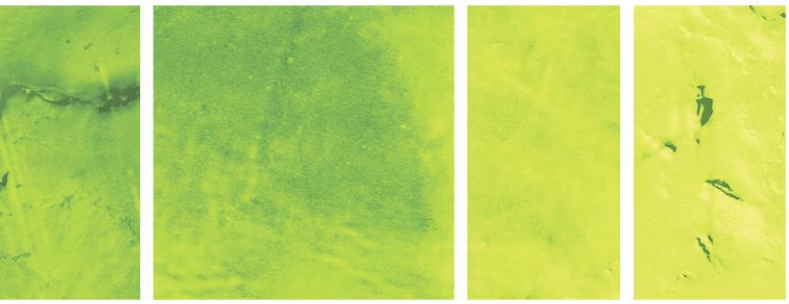
Competitive advantage trials

Virus replication and shedding of K5 and the Czech variant was compared in 4-5 week old rabbits, to assess their potential to shape RHDV epidemiology by their ability to shed and transmit to bystander rabbits. Kittens were susceptible to infection with virus doses as low as 10 ID_{50}, had low-level viremia and shed virus in their faeces for up to five days, and could transmit virus to bystanders before they seroconverted. Notably, the K5 variant showed non-significantly lower growth, shedding, transmission and pathogenicity compared to the Czech variant in kittens.

At Day 5 post infection quantitative RT-PCR was used to analyse virus loads in various tissues. Virus was found in all tissues examined and in quantities several orders of magnitude higher than observed in control animals infected with inactivated virus, clearly indicating virus replication in kittens. The median tissue titres of the Czech virus were higher than the K5 titres in most tissues, with the exception of the bile, although the difference was not significant. Interestingly, as seen for swab samples, bile and duodenum titres did not correlate with the virus level observed in blood and other tissues. Compared to adult rabbits that succumb to RHDV infection, virus titres in the kitten livers were low, and final tissue titres of virus in dead animals are not dependent on the inoculation dose or time post infection.

Transmission experiments

Of the experimentally-infected animals (high dose), all except one became infected (#103, Figure 4i), and one of the experimentally-infected animals died (#96). In replicate 1, two of the four bystander rabbits became infected, one with K5 and one with a mixture of both variants (Figure 4g). The virus mixture in the liver of this animal consisted mainly of K5 (> 90%). In replicate 2, only one of the bystander rabbits became infected, with K5 (Figure 4h). Replicate 3 revealed three bystander rabbits infected with the Czech variant (Figure 4i), which likely reflects the higher amount of virus shed by the infected animal that died in this cage (Figure 4c+f). Furthermore, one of the bystander rabbits was found dead four days after the addition of the infected kittens (day 6 of the experiment).



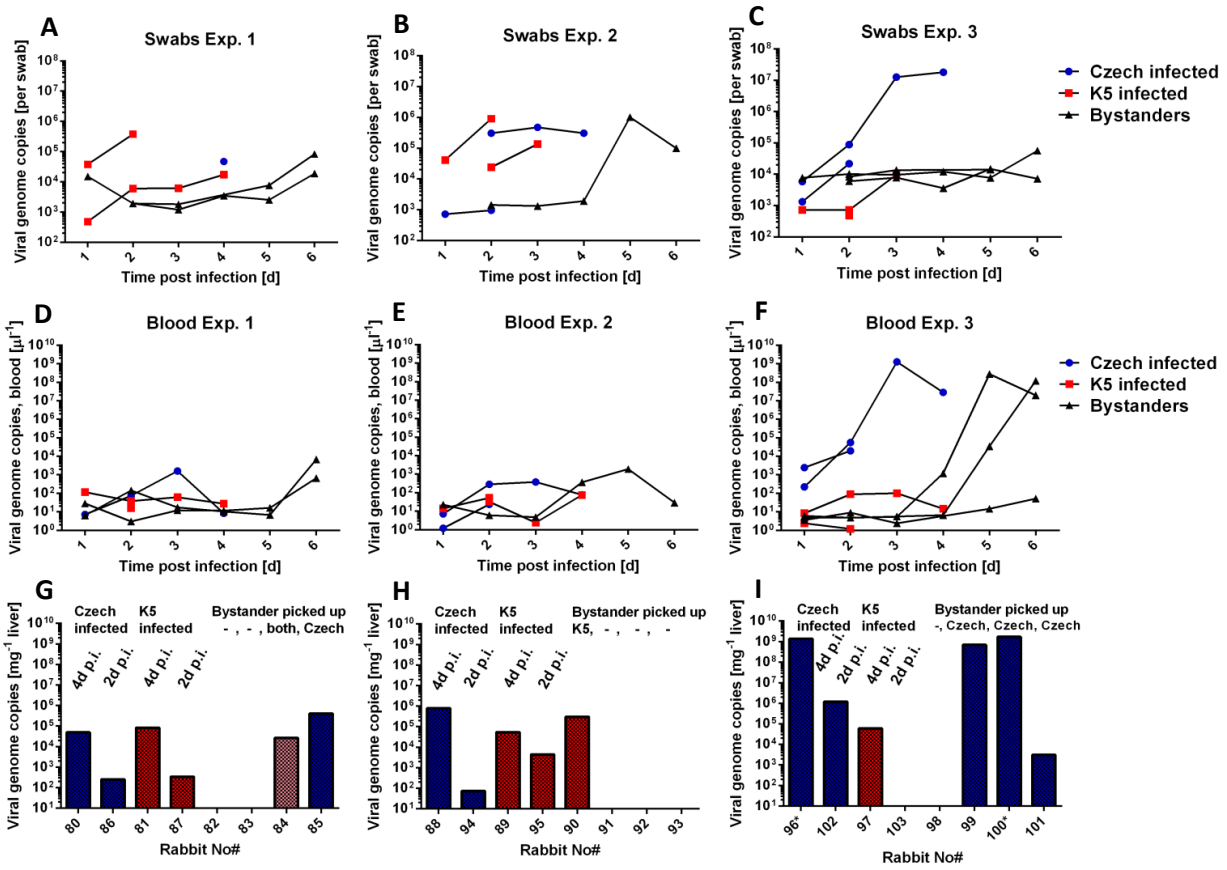


Figure 4: Transmission of the Czech variant and K5 from kittens infected with a high dose to littermates. Upper panel (A,B,C): Virus in swab rectal swabs of kittens infected with the Czech variant (blue) or K5 (red) and bystanders (black); Middle panel (D,E,F): Virus concentration in blood samples of infected and bystander kittens (colour code as above). Lower panel (G,H,I): Virus load in livers of infected kittens and bystanders at the time of autopsy at Day 4 and Day 2 post infection (infected kittens) and Day 5 (bystanders) (colour code as above). The purple bar in panel G indicates a mixed infection with both variants.

Discussion

This project represents the first direct comparison of variants of RHDV for use as biological control agents. RHDV variants were collected from Western Europe, South Korea, China and South Australia (see Table 2).

Overcoming immunity to RHDV antibodies

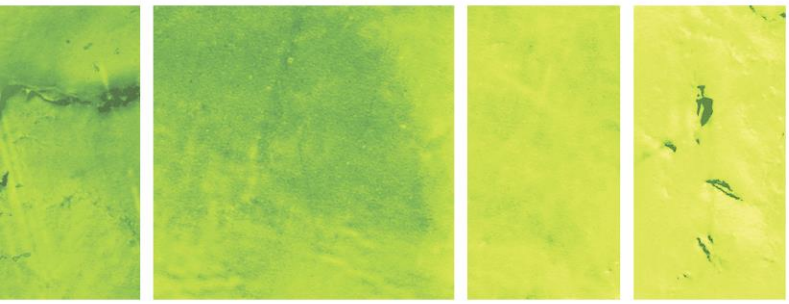
The selected variants of RHDV were tested in rabbits previously vaccinated with the RHDV vaccine available in Australia (Cylap®). It was found that, for all the selected variants except X15, the infectious dose required for infection in vaccinated rabbits was more than 1000 times the dose required in naïve rabbits. Unfortunately this result indicates that it was not possible to identify a virus that is superior based on the capacity to over come antibodies against RHDV. Perhaps unexpectedly, the C1 variant was not able to overcome previous vaccination with Cylap®. This variant had reportedly been collected from vaccinated commercial rabbits, although this vaccine had been derived from an RHDVa variant, not the classical RHDV. X15 did provide interesting results in that between 14 and 28% of rabbits with RHDV antibodies died at all virus doses tested. X15 belongs to the RHDV2 group of viruses, which is a new group of viruses. A distinctive phenotypic property of RHDV2 is its host susceptibility. RHDV only cause disease in the European rabbit, and no other American, Asian or European lagomorphs of the genus *Romerolagus*, *Lepus* and *Sylvilagus* have been shown to be naturally or experimentally susceptible to it. By contrast, RHDV2 has been implicated as the cause of several outbreaks of acute hepatitis in rabbits and Cape hares (*Lepus capensis mediterraneus*) in Sardinia. This is the first report of a lagovirus causing fatal hepatitis in both rabbits and hares (Puggioni *et al*. 2013). Despite the host range including only lagomorphs, this broader host specificity may make RHDV2 less attractive as a biological control agent due to concerns about the potential of the virus to infect non-target species.

Overcoming genetic resistance and RCV-A1 mediated cross-protection

The K5 variant was shown to be more infectious in wild rabbits from Bulloo Downs when compared to Tur09 and the Czech variant. This result is important, as it indicates that fewer infectious particles of K5 are required to infect and kill these rabbits compared to the Czech variant, and, at least in a population of wild rabbits that are similar to those from Bulloo Downs, the K5 variant should be capable of spreading more readily to infect and kill rabbits. The K5 variant also appeared to be the variant least affected by the presence of antibodies to the benign calicivirus RCV-A1, with only 2 times the RID_{50} required to infect rabbits, compared to 13.5 times for the K9 variant, 18 times for the E9 variant and 100 times for the Czech variant. Based on this information, both the K5 and K9 variants would be more suitable for use as a biological control agent in rabbit populations with a high prevalence of RCV-A1 antibodies than the current Czech variant, with the K5 variant the preferred agent.

Transmission experiments in young rabbits

It is an important finding that, despite low levels of replication and lack of disease, RHDV can be shed from kittens and infect bystander rabbits, confirming that they may play a role in RHDV epidemiology in the field. The importance of this role is in comparison to the virus shed and transmitted by dead adult rabbits needs to be determined. The detailed quantitative



data on the infection dynamics of RHDV in very young rabbits collected here will facilitate determining this contribution of kittens to RHDV epidemiology. The K5 variant did not show a competitive advantage in kittens compared to the Czech variant. In adults however, K5 infection did result in increased mortality rates in rabbits compared to the Czech variant, and it also killed animals faster. With respect to a potential release of K5, this combination may in fact be desirable. A virus that infects kittens less effectively may lead to fewer young animals being exposed during an outbreak and subsequently developing antibodies. This may reduce the number of immune animals within the breeding population. Additional experiments are needed to confirm that this is indeed the case for K5.

Towards a release

The decision to release a new RHDV variant will primarily depend on the confidence we have that:

a) new virus variants can be established in an environment where other closely related variants are already circulating, and,

b) new virus variants will increase mortality rather than rapidly attenuating to form less virulent variants.

c) new virus variants do not increase non-lethal infection of young rabbits.

Previous experience with releases of additional variants of myxoma virus (MV) showed that a variant can be successfully introduced to the field if rabbits are inoculated shortly before field variants of virus spread naturally, but that establishment of that variant in the field can be unsuccessful (Fenner *et al*. 1957, Merchant *et al*. 2003, Berman *et al*. 2006). This may also be a problem in establishing new RHDV variants. Short-term success in specific localities seems likely if new variants are released ahead of expected field-variant outbreaks, yet general persistence may be more difficult to achieve. MV attenuated to form less virulent variants within a few years of release in both Australia and Europe (Fenner and Fantini 1999) however the same has not been seen with RHDV. The lack of such obvious change in RHDV suggests that there must be strong natural selection of variants that cause high mortality; generally the virus must be evolving to maximise its capacity to infect and spread among increasingly resistant rabbits in a 'biological arms race'. Indeed, the emergence and subsequent outbreaks due to variants in Europe and Asia clearly indicate that these variants do persist at a broad-scale population level.

In the second of the two likely scenarios above, it is envisaged that a dynamic equilibrium will be established between resistance and virulence and so in the long term, RHDV will remain as a mortality agent helping to control rabbits even if it loses some of its initial effectiveness. This seems to be the picture for MV where, despite initial attenuation, variants of the virus still kill between 40–60% of infected rabbits even 60 years after release in Australia.

There is now some evidence that RHDV in Australia is evolving to maintain very high virulence and maintain its capacity to spread. Information provided above shows that the field collected virus Tur09, collected from Turretfield, was highly infectious in Bulloo Downs rabbits compared with Czech virus. Nonetheless, K5 performed better than Tur09 in Bulloo rabbits that had previously been selected for resistance to the Czech variant. These data indicate that, even in the absence of spread and establishment in the field, the K5 variant

would prove to be a superior biocide in these wild rabbit populations when compared to the Czech variant.

Recommendations for making releases

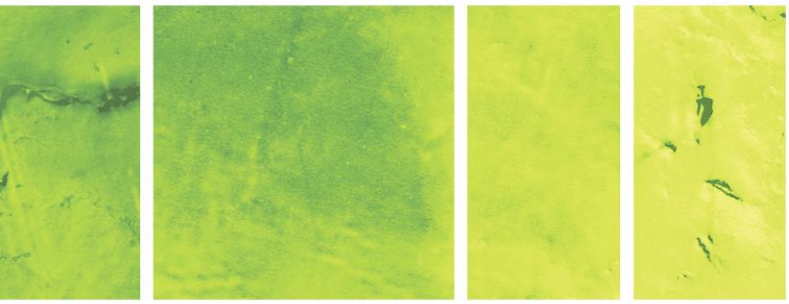
Because RHDV is already well established in Australia and by now is well adapted to field conditions, it may be difficult to establish new variants. Releases of new variants will have to be coordinated via a national release strategy. Releases of new variants should be made just prior to the anticipated natural spread of RHDV in the field and should be made by infecting as many rabbits as possible. Delivery on carrot or oat baits seems most cost-efficient for this purpose (Mutze *et al*. 2010b). RHD outbreaks do not occur at the same time of year everywhere in Australia so releases will need to take this variation into account.

There will be major costs associated with releasing a new virus variant on a scale sufficient for it to have a chance of establishing in the presence of locally-adapted field variants. Consequently, it will be important to have a clear understanding of where the new virus might be most useful and the likely economic and ecological benefits that should follow release. For example, a new variant that was able to cause acute disease in rabbits that had antibodies to RCV-A1 would be most useful in those areas of Australia where RCV-A1 is present. Implementation costs would need to be weighed against likely industry benefits which, in that particular instance, are likely to be high given that the fine-wool industry is centred on the same areas affected by this interfering benign virus (Vere *et al*. 2004).

The results of the above trials indicate that:

- There is evidence that the K5 variant requires over 30 times less virus to infect wild Bulloo Downs rabbits than the Czech variant.
- The K5 variant appears to be able to overcome the partial protection offered by previous infection with RCV-A1, as does the K9 and E9 variants to a lesser extent.
- None of the overseas variants was highly infectious to rabbits with RHDV antibodies. X15 did show a non-dose dependent ability to infect and kill some rabbits with RHDV antibodies.

Based on these data, the K5 variant appears to be the most promising of the candidate variants tested, and has demonstrated advantages over the Czech variant currently available in Australia. This variant appears suitable as a biological control agent and warrants field investigation. We recommend that this variant should be selected as an agent for release in Australia. The ability to better overcome cross protection provided by the endemic benign virus RCV-A1 is likely to make this variant invaluable.



Animal use

All procedures involving animals were carried out according to the 'Australian Code of Practice for the Care and Use of Animals for Scientific Purposes' and were approved by:

- CSIRO Ecosystem Sciences Animal Ethics Committee (CESAEC# 11-01),
- Elizabeth McArthur Agricultural Institute Animal Ethics Committee (#M10/09 and #M11/09), and
- Orange Animal Ethics Committee (ORA 11/14/001).

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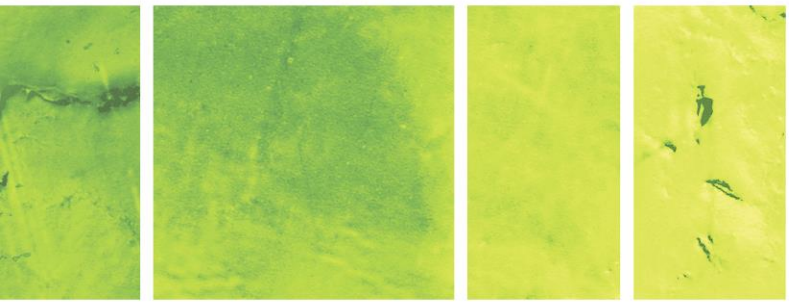

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