

## Survival of rabbit haemorrhagic disease virus (RHDV) in the environment

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(Accepted 23 March 2004)

### SUMMARY

A study was conducted to investigate the persistence of rabbit haemorrhagic disease virus (RHDV) in the environment. Virus was impregnated onto two carrier materials (cotton tape and bovine liver) and exposed to environmental conditions on pasture during autumn in New Zealand. Samples were collected after 1, 10, 44 and 91 days and the viability of the virus was determined by oral inoculation of susceptible 11- to 14-week-old New Zealand White rabbits. Evidence of RHDV infection was based on clinical and pathological signs and/or seroconversion to RHDV. Virus impregnated on cotton tape was viable at 10 days of exposure but not at 44 days, while in bovine liver it was still viable at 91 days. The results of this study suggest that RHDV in animal tissues such as rabbit carcasses can survive for at least 3 months in the field, while virus exposed directly to environmental conditions, such as dried excreted virus, is viable for a period of less than 1 month. Survival of RHDV in the tissues of dead animals could, therefore, provide a persistent reservoir of virus, which could initiate new outbreaks of disease after extended delays.

### INTRODUCTION

Rabbit haemorrhagic disease virus (RHDV) emerged in China in 1984 and spread throughout Europe over the rest of the decade. The virus causes a severe, systemic disease in European rabbits (*Oryctolagus cuniculus*), which is characterized by hepatocellular necrosis and disseminated intravascular coagulation. Morbidity of 100% and mortality of 90–95% are observed in rabbits older than 3 months of age [1–3]. In addition to a natural resistance of young rabbits (up to 4 weeks of age) to this disease [4–6], maternal antibodies provide protection to an age of approximately 8 weeks [7].

In most countries, research into RHDV has focused on developing methods to minimize the effects of the virus on wild, farmed and pet rabbits. However, in Australia and New Zealand, where the introduced European rabbit is a major vertebrate pest species, research has focused on finding methods to maximize the effect of the virus on wild rabbit populations so that the virus can be used as a biological control agent.

The limited knowledge on the survival of RHDV in the natural environment has been inferred from epidemiological evidence based on continuous infection and mortality rates within wild rabbit populations [8], and the detection of virus in rabbits, flies and fly spots [9]. In laboratory-based studies, Smid and colleagues [10] investigated the survival of RHDV at various temperatures and McColl and colleagues [11] reported that RHDV remains infective in rabbit carcasses up to 30 days post-death. However, there have been no

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published studies of the biological stability of RHDV under natural environmental conditions.

The study reported in this paper, the first in a series of two, concerns the influence of environmental conditions on the survival of RHDV and on the response in rabbits to virus exposed to the environment. The aim was to gain a better understanding of the field epidemiology of this virus in its natural host by determining the duration of RHDV infectivity following exposure to typical rural environmental conditions in New Zealand. Two different carrier materials were impregnated with the virus and held in a natural environment for up to 91 days. Daily temperature and humidity values were recorded over this time period and the infectivity of the samples was measured at various time intervals by inoculation into susceptible rabbits.

## MATERIALS AND METHODS

### Virus

The commercial product 'RCD-ZEN' (Zenith Technology Corp. Ltd, Dunedin, New Zealand) generated from RCD CAPM V-351 (Czechoslovakian strain) Master Seed Virus was used. The batch purchased for this study (Z25) had a rabbit LD<sub>50</sub> titre of between 10<sup>6</sup> and 10<sup>7</sup> per ml (M. Shepherd, personal communication).

### Preparation of viral suspension and its exposure to the environment

Two vehicles were used to study RHDV survival: cotton tape and bovine liver. The cotton sample was prepared by absorbing viral suspension (0.5 ml per rabbit) onto a 12-cm piece of washed, sterilized 100% cotton tape (Trendy Trims Ltd, Onehunga, Auckland, New Zealand) and leaving to dry. The liver sample was prepared by injecting viral suspension (0.5 ml per rabbit) into a 20-g piece of bovine liver. Untreated cotton and bovine liver samples were used as negative controls.

A special unit was designed to allow exposure of samples to environmental conditions while protecting them from damage caused by insects and animals. The units consisted of a wooden-framed box with walls of insect-proof netting (Fibreglass Flyscreen Mesh, Ulrich Aluminium Ltd, Manakau City, New Zealand) and a pitched roof made of clear corrugated plastic sheets with a low ultraviolet (UV) absorption

rating (Sunlight Light Blue, Suntuf Inc., Kutztown, PA, USA). The roof protected samples from rain-water but allowed passage of UV light. Within the sampling unit, samples of virus-impregnated cotton tape and bovine liver were placed in open 23 × 13 cm plastic racks and the racks placed in metal cages suspended on metal chains 15 cm above the ground. Control samples were placed in an identical sampling unit and both units placed in an open pasture environment.

To recover virus after exposure to the environment, each cotton tape and liver sample (diced) was placed in a 200-ml container, covered with resuspension medium (1 part distilled water to 4 parts serum-free Eagle's medium, 2.5 ml per rabbit to be inoculated) and left to stand at 4 °C for 1 h [12]. The samples were then centrifuged at 1800 *g* for 15 min and supernatants collected and filtered through 0.45- $\mu$ m filters (Minisart, Sartorius Australia Ltd, Oakleigh, Victoria, Australia) followed by filtration through 0.2- $\mu$ m filters to remove cobacteria and fungi.

### Rabbits

Eleven- to 14-week-old New Zealand White rabbits, which had not been vaccinated against RHDV, were obtained from a single commercial laboratory animal colony. Virus-inoculated rabbits were kept in a climate controlled room (17 °C) and negative control rabbits were housed in a separate building on the same site. One uninoculated sentinel rabbit per trial was housed in the same room as the virus-inoculated rabbits to test for horizontal transmission of the virus. The distance between the sentinel and the RHDV-inoculated rabbits was approximately 30 cm.

Each rabbit was individually housed in a standard rabbit cage (56 × 44 × 45 cm) and fed *ad libitum* with commercial pelleted rabbit feed. Rabbits were acclimatized for 5–7 days prior to inoculation. Following inoculation, rabbits were observed continuously for 7 days, then at 4-h intervals until 10 days post-inoculation (p.i.) and then at daily intervals until 30 days p.i. Body weight was recorded prior to inoculation, at 5, 10, 20 and 30 days p.i. and immediately post-death.

Our previous studies showed that deaths from rabbit haemorrhagic disease (RHD) usually occurred 2–3 min after the onset of neurological signs. As soon as these neurological signs were observed, rabbits were anaesthetized with an intramuscular injection of ketamine hydrochloride (100 mg/ml; Phoenix Pharm Distributors Ltd, Auckland, New Zealand)

and xylazine (20 mg/ml; Phoenix Pharm Distributors Ltd), and then euthanized by intracardiac injection of sodium pentobarbitone (Pentobarb 300, 300 mg/ml; National Veterinary Supplies Ltd, Auckland, New Zealand). Rabbits that survived 30 days p.i. were anaesthetized and euthanized as above. Necropsies were performed on all rabbits and gross pathological observations recorded.

A rabbit was classified as being infected with RHDV (and thus, to have been inoculated with infectious virus) if it showed clinical signs typical of RHD (apathy, dullness, ocular haemorrhaging and cyanoses of mucous membranes, ears and eyelids, anorexia, increased respiratory rate, convulsions, ataxia, posterior paralysis); or showed pathological changes typical of RHD (pale yellow or greyish liver with marked lobular pattern, petechial and echymotic, multifocal haemorrhages of the lung, lung oedema, lung congestion, splenomegaly, poor blood coagulation, swollen, dull, pale to patchy reddish discolouration of the kidney); or tested positive for RHDV antibodies with a 1:40 dilution of serum.

We concluded that if none of these criteria were met the rabbit was uninfected and the virus in the inoculum was inactivated.

### RHDV antibody testing

Blood samples were collected from ear veins 3–5 days prior to inoculation of the rabbit, at 5, 10, 20 and 30 days p.i., and also from euthanized and dying rabbits. Blood samples were centrifuged at 1800 g for 15 min and the sera removed and stored at  $-80^{\circ}\text{C}$  until testing. Antibodies to RHDV were assayed by AgResearch (Wallaceville Animal Research Centre, Upper Hutt, New Zealand) using a competition ELISA [13]. Samples were assayed in fourfold serial dilutions from 1:10 to 1:640. Samples were classified as RHDV positive if inhibition was  $\geq 50\%$  in serum diluted 1:40.

### Study design

The study comprised two short-term pilot experiments to develop the methodology followed by a long-term exposure trial. The objectives of the pilot experiments were to define the viral dosages and the route of inoculation to be used, to determine the suitability of the carrier materials, and to minimize the number of rabbits required in the subsequent long-term trial.

Table 1. Number of rabbits showing disease or seroconversion to rabbit haemorrhagic disease virus (RHDV) following intramuscular injection with virus preparations from inoculated cotton tape and bovine liver held at  $4^{\circ}\text{C}$  for 24 h

Carrier material	Dilution	No. of rabbits infected*/inoculated
Cotton	$10^0$	2/2†
	$10^{-2}$	2/2†
	$10^{-3}$	0/2
Liver	$10^0$	2/2†
	$10^{-2}$	2/2†
	$10^{-3}$	0/2
Control	—	0/4

\* Infection defined as presence of clinical signs and/or pathology typical of RHD and/or seroconversion (RHD antibody titre  $\geq 1:40$  on one or more sampling occasions).

† One rabbit had RHD and one rabbit seroconverted without signs of RHD.

### Pilot studies

Pilot experiment 1 was conducted under laboratory conditions. Virus-impregnated cotton tape and bovine liver, were prepared as described above and stored at  $4^{\circ}\text{C}$  for 24 h. Virus was recovered from the carrier materials and two dilutions ( $10^{-2}$  and  $10^{-3}$ ) were prepared in serum-free Eagle's medium. For each of the three treatments (undiluted,  $10^{-2}$  and  $10^{-3}$ ) two rabbits were inoculated by intramuscular inoculation. Negative control suspensions prepared from both carrier materials were inoculated into two rabbits each (Table 1). Rabbits were monitored for up to 30 days p.i. and assessed for RHDV infection as above.

For pilot experiment 2, virus was impregnated on each of the carrier materials, as described above, and placed for up to 5 days in a sampling unit located in a rural environment near Dannevirke (longitude 176.095, latitude  $-40.214$ ) in the North Island of New Zealand. Control samples of cotton tape and bovine liver were placed in a second unit located 10 m away. Viral suspensions were prepared from cotton-tape samples removed after 1 and 5 days and bovine liver samples removed after 5 days. Rabbits were inoculated orally by syringe with 1 ml of either an undiluted or a  $10^{-2}$  dilution of the viral suspension or with a control preparation. Table 2 shows the number of rabbits inoculated with each suspension.

In addition, the intramuscular and oral inoculation routes were compared by inoculating three

Table 2. Number of rabbits showing disease or seroconversion to rabbit haemorrhagic disease virus (RHDV) following oral dosing with virus preparations from inoculated cotton tape and bovine liver held in the environment for 1 or 5 days

Carrier material	Duration of exposure	Dilution	No. of rabbits infected*/inoculated
Cotton	24 h	10 <sup>0</sup>	4/4
		10 <sup>-2</sup>	3/3
		Control	0/2
	5 days	10 <sup>0</sup>	4/4
		10 <sup>-2</sup>	3/3
		Control	0/2
Liver	5 days	10 <sup>0</sup>	4/4†
		10 <sup>-2</sup>	3/3‡
		Control	0/2

\* Infection defined as presence of clinical signs and/or pathology typical of RHD and/or seroconversion (RHD antibody titre  $\geq 1:40$  on one or more sampling occasions).

† One rabbit was seropositive at time of death (5 days p.i.).

‡ Two rabbits had RHD and one rabbit seroconverted without signs of RHD.

and two rabbits respectively with 0.5 ml of virus suspension that had been held at 4 °C for 24 h on cotton tape.

#### Long-term exposure study

The virus-impregnated cotton tape and bovine liver were placed in a sampling unit located in an open pasture environment near Himatangi (longitude 175.317, latitude -40.400) in the North Island of New Zealand. Control samples of bovine liver and cotton tape were kept in a unit placed in the same environment 10 m from the sample unit. Samples were removed at 1, 10, 44 and 91 days after placement. Rabbits were inoculated orally with 1 ml of either undiluted or a 10<sup>-2</sup> dilution of viral suspension prepared from the samples or with control preparation. Table 3 shows the sampling intervals and the number of rabbits inoculated with each dilution. If virus-inoculated rabbits were clinically unaffected after exposure to a given treatment, the subsequent exposure interval was still evaluated for that treatment. Failure to observe clinical disease in two consecutive exposures was considered confirmation of virus inactivation for a given treatment. For each sampling interval an uninoculated sentinel control rabbit was caged in the same room with the virus-inoculated control rabbits.

Table 3. Number of rabbits showing disease or seroconversion to rabbit haemorrhagic disease virus (RHDV) following oral dosing with virus preparations from inoculated cotton tape and bovine liver held in the environment for 1, 10, 44 and 91 days

Carrier material	Duration of exposure	Dilution	No. of rabbits infected*/inoculated
Cotton	1 day	10 <sup>0</sup>	4/4†
		10 <sup>-2</sup>	4/4
		Control	0/2
	10 days	10 <sup>0</sup>	4/4
		10 <sup>-2</sup>	0/4
		Control	0/2
	44 days	10 <sup>0</sup>	0/4
		10 <sup>-2</sup>	0/4
		Control	0/2
	91 days	10 <sup>0</sup>	0/4
		Control	0/2
		Control	0/2
Liver	10 days	10 <sup>0</sup>	4/4
		10 <sup>-2</sup>	3/4‡
		Control	0/2
	44 days	10 <sup>0</sup>	3/4
		10 <sup>-2</sup>	1/4§
		Control	0/2
	91 days	10 <sup>0</sup>	4/4
		10 <sup>-2</sup>	0/4
		Control	0/2

\* Infection defined as presence of clinical signs and/or pathology typical of RHD and/or seroconversion (RHD antibody titre  $\geq 1:40$  on one or more sampling occasions).

† One rabbit seropositive (titre  $\geq 1:640$ ) at time of death (4 days p.i.).

‡ One rabbit seropositive (titre  $\geq 1:640$ ) at 5 days p.i.

§ This rabbit seroconverted (titre  $\geq 1:640$ ) without signs of RHD at 10 days p.i.

#### Weather data recording

Gemini data loggers were placed in the sampling units described above and the temperature and relative humidity recorded at 2-min intervals. Gemini Logger Manager Version 2.10 (Hastings Data Loggers, Port Macquarie, NSW, Australia) was used to download the data. These climate data were summarized to hourly averages and the temperature-humidity indexes [14], averages, maximums and ranges for temperature and humidity were calculated for the different intervals of viral exposure.

#### Data analysis

Counts of surviving and dying rabbits in different treatment groups were compared using the exact

estimation method in SPSS version 9.0 (SPSS Inc., Chicago, IL, USA). When multiple pairwise comparisons were conducted, the Bonferroni correction was applied [15]. The time interval to death was investigated with Kaplan–Meier Survival Plots and compared statistically between groups using log rank tests in S-Plus for Windows version 2000 (Insightful Corp., Seattle, WA, USA).

## RESULTS

All rabbits used in the three experiments tested seronegative prior to the commencement of each study.

### Pilot experiment 1

All rabbits inoculated with  $10^0$  or  $10^{-2}$  virus preparations from either of the carrier materials showed signs of viral infection or seroconverted (Table 1). Four of the eight rabbits which received these preparations died with typical RHD signs and the remaining four rabbits tested positive for RHDV antibodies on at least one sampling occasion, with titres ranging from 1:160 to  $\geq 1:640$ . None of the rabbits inoculated with  $10^{-3}$  virus preparation or with the control preparation from either carrier material showed signs of RHD or seroconverted. No clinical signs or seroconversion were observed in the uninoculated sentinel rabbit kept in the same room as the inoculated rabbits.

### Pilot experiment 2

All rabbits that were inoculated orally with undiluted or  $10^{-2}$  preparations from the virus-impregnated cotton tape that had been held in the environment for 1 or 5 days, died with typical signs of RHD (Table 2). Six of the seven rabbits inoculated with preparations from the virus-impregnated liver that had been held in the environment for 5 days, died from RHD. The remaining rabbit (inoculated with  $10^{-2}$  dilution) did not show signs of RHD but had an antibody titre of  $\geq 1:640$  at 30 days p.i. The rabbits inoculated with control preparations derived from either carrier material, and the same-room sentinel rabbit, did not show signs of RHD and were not RHDV-antibody positive at any time.

All rabbits inoculated intramuscularly (3) or orally (2) with undiluted virus preparation, which had been held at 4 °C for 24 h on cotton tape, showed clinical signs of RHD and died or were euthanized (data not presented).

## Long-term exposure study

### Cotton tape

All rabbits that were inoculated orally with undiluted or  $10^{-2}$  preparations from the virus-impregnated cotton tape that had been held in the environment for 1 day, developed RHD (Table 3). For preparations made after 10 days of environmental exposure, only the rabbits that received the undiluted preparation were infected, and none of those that received the  $10^{-2}$  preparation showed signs of RHD or seroconverted. For preparations made after 44 and 91 days of environmental exposure, none of the rabbits, which received either undiluted or diluted preparations showed signs of RHD or seroconverted.

### Bovine liver

For the preparations eluted from bovine liver, infectious virus was still present in samples that had been exposed to the environment for 91 days. All four rabbits inoculated with the undiluted preparation at that sampling time developed RHD (Table 3). However, none of the four rabbits inoculated with the diluted ( $10^{-2}$ ) preparation from 91 days exposure were infected. At 44 days exposure, three of the four rabbits inoculated with undiluted preparations developed RHD, but only one of the four rabbits that received the diluted preparation became infected. Rabbits inoculated with control preparations derived from either carrier material did not produce any signs of RHD or other diseases, and were not RHDV-antibody positive at any sampling occasion. None of the same-room sentinel rabbits developed RHD symptoms or were seropositive at any time.

For the cotton samples there was a significant association between duration of environmental exposure and risk of death in both the diluted ( $P=0.006$ ) and the undiluted groups ( $P<0.001$ ). Multiple pairwise comparisons for risk of death between different exposure times of cotton samples were not significant. The risk of death was higher ( $P=0.029$ ) for rabbits inoculated with undiluted *vs.* diluted samples eluted from cotton tape after exposure to the environment for 10 days. There was a significant association between duration of environmental exposure of liver samples and risk of death among rabbits inoculated with diluted ( $10^{-2}$ ) preparations ( $P=0.055$ ). Because RHDV from liver samples in the high-dilution group was still infective after 91 days of environmental exposure, it was not possible to estimate the duration of infectivity in this



Table 4. Antibody titres and time of death (in hours) of rabbit haemorrhagic disease virus (RHDV)-inoculated rabbits

Study	Material	Exposure	Virus dilution	Time to death	RHD death	Highest level of RHDV antibodies p.i.			
						5 d	10 d	20 d	30 d
PS 1	Cotton	4 °C, 1 day	10 <sup>-2</sup>	n.a.		≥1:640	Neg.	Neg.	Neg.
PS 1	Cotton	4 °C, 1 day	10 <sup>0</sup>	n.a.		Neg.	Neg.	≥1:640	≥1:640
FS	Liver	Env., 44 days	10 <sup>-2</sup>	n.a.		Neg.	≥1:640	≥1:640	≥1:640
PS 1	Liver	4 °C, 1 day	10 <sup>0</sup>	n.a.		Neg.	1:40	≥1:640	≥1:640
PS 1	Liver	4 °C, 1 day	10 <sup>-2</sup>	n.a.		1:40	1:40	≥1:640	≥1:160
PS 2	Liver	Env., 5 days	10 <sup>-2</sup>	n.a.		1:40	≥1:640	≥1:640	≥1:640
FS	Cotton	Env., 1 day	10 <sup>0</sup>	98 h	≥1:640				
PS 2	Liver	Env., 5 days	10 <sup>0</sup>	126 h	n.s.	1:40			
FS	Liver	Env., 10 days	10 <sup>-2</sup>	165 h	n.s.	≥1:640			

PS, pilot study; FS, final study; Neg, negative; Env., environmental placement; n.s., not sampled; n.a., not applicable.

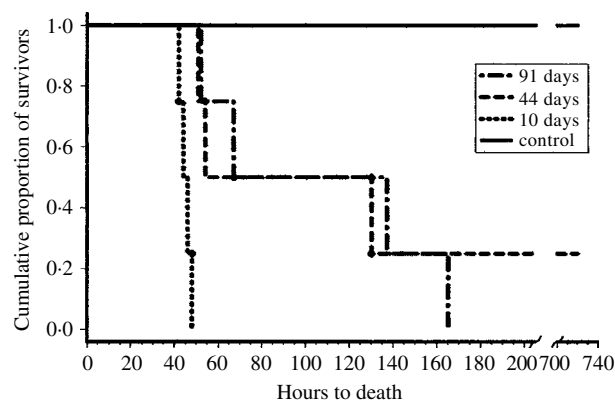


Fig. 1. Kaplan-Meier survival plot for hours to death of rabbits inoculated with a 10<sup>0</sup> RHDV dilution prepared from liver after exposure to environmental conditions. Log rank test comparing survivorship among virus-inoculated rabbits = 14, D.F. = 2,  $P=0.0009$ .

group. The failure to detect differences between dilutions within the liver treatment groups at the  $P=0.1$  threshold for statistical significance for the shorter exposure periods is attributed to the small sample sizes.

### Serology

Out of 87 virus-inoculated rabbits used in the three studies, only nine tested positive for antibodies to RHDV on one or more sampling occasions (Table 4). Only three out of 40 animals sampled at the time of death or shortly before death had antibodies against RHDV at that time. The six other seroconverting rabbits survived RHDV infection and of these rabbits, three were seropositive at 5 days p.i. The antibody titres of two of these rabbits increased between 5 and

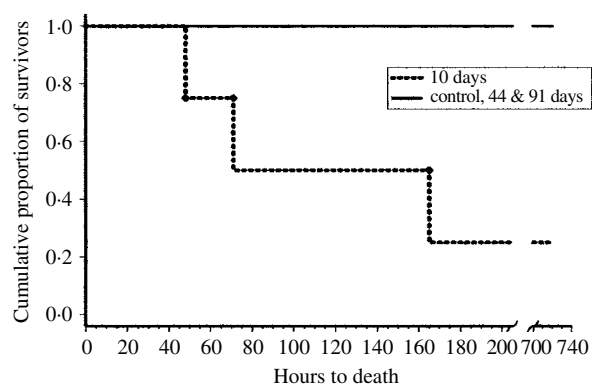


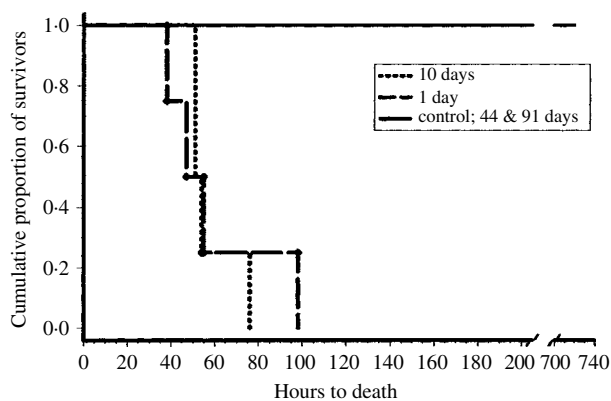
Fig. 2. Kaplan-Meier survival plot for hours to death of rabbits inoculated with a 10<sup>-2</sup> RHDV dilution prepared from liver after exposure to environmental conditions. Log rank test comparing survivorship among virus-inoculated rabbits = 8.3, D.F. = 2,  $P=0.0158$ .

20 days, with the third rabbit becoming antibody negative at 10 days p.i. and remaining negative at 20 and 30 days p.i. The other three surviving rabbits seroconverted at 10 or 20 days p.i. The titres and sampling times are shown in Table 4.

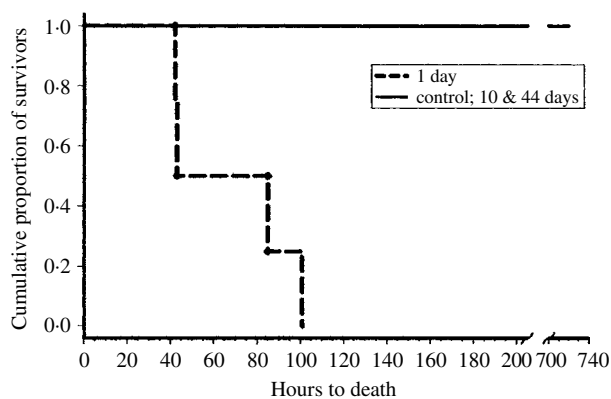
### Survival analysis

Continuous monitoring of rabbits in the post-inoculation period allowed the exact hour of death to be recorded. The survival times of inoculated rabbits are compared in Figures 1-4.

Time to death was significantly prolonged in rabbits inoculated with virus that had been exposed to environmental conditions for longer. As the periods of liver sample exposure to the environment increased from 10 to 44 to 91 days, so did the time to death for



**Fig. 3.** Kaplan–Meier survival plot for hours to death of rabbits inoculated with a  $10^0$  RHDV dilution derived from cotton tape after exposure to environmental conditions. Log rank test comparing survivorship among virus-inoculated rabbits = 17, D.F. = 3,  $P = 0.0007$ .



**Fig. 4.** Kaplan–Meier survival plot for hours to death of rabbits inoculated with a  $10^{-2}$  RHDV dilution derived from cotton tape after exposure to environmental conditions. Log rank test comparing survivorship among virus-inoculated rabbits = 14, D.F. = 2,  $P = 0.0009$ .

the rabbits treated with the respective inocula of undiluted ( $10^0$ ) RHDV ( $P < 0.001$ , Fig. 1). Inoculation of rabbits with diluted virus from liver samples kept from 10 to 44 days in the environment (Fig. 2) also extended the time to death ( $P < 0.016$ ). Cotton samples kept for longer intervals (10, 44 and 91 days for undiluted virus samples and 1, 10 and 44 days for diluted virus samples) resulted in prolonged time to death for both the undiluted (Fig. 3,  $P < 0.001$ ) and diluted (Fig. 4,  $P < 0.001$ ) inocula.

### Environmental conditions

The long-term exposure study was conducted from 25 March 2001 until 24 June 2001. During this period mean ambient temperatures declined (Table 5) and

**Table 5.** Mean daily temperature ( $T$ ) and humidity ( $H$ ) recordings for different periods of RHDV exposure to the environment

Interval	$T_{av}$ (°C)	$T_{av}$ range (°C)	$H_{av}$ (%)	$H_{av}$ range (%)
1 day	17.6	13.4	80.4	22.5
> 1–10 days	15.2	12.6	74.6	24.6
> 10–44 days	12.9	13.1	85.5	22.2
> 44–91 days	8.8	8.8	91.6	20.4

$T_{av}$ , average temperature;  $H_{av}$ , average humidity.

the diurnal temperature range decreased by approximately 40% between days 1 and 90. Relative humidity increased during the course of the study, although the diurnal humidity range remained largely stable.

Temperature and humidity values for daytime (06:00–18:00 hours) and night-time (18:00–06:00 hours) are shown in Table 6. Temperature range was considerably broader during the daytime at the beginning of the virus exposure study, but declined to similar levels for day- and night-time by the end of the study. Relative humidity was generally higher at night, and the total range and average range of humidity was greater during the day. Humidity increased as the Southern Hemisphere winter months approached.

Figure 5 compares the daytime and the night-time range of the Temperature–Humidity Index. In March and April 2001 the fluctuation of the daytime index was considerably larger than at night time. The range of the index in May and June 2001 followed similar patterns during the day and night with lower values than in the previous months.

### DISCUSSION

The ability of agents to survive in the environment is a key determinant of the epidemiology of infectious diseases and of the relative importance of potential routes of transmission. At the commencement of this study, there was little published information available on the survival characteristics of RHDV, although data on related caliciviruses suggested that prolonged survival under environmental conditions was likely [16–18]. The lack of methods for *in vitro* cultivation of RHDV presents a major obstacle to understanding the ability of this virus to endure under field conditions. Options for studying survival characteristics

Table 6. Mean daytime and night-time temperature ( $T$ ) and humidity ( $H$ ) recordings for different periods of RHDV exposure to the environment

Virus exposure	$T_{av}$ (°C)		$T_{av}$ range (°C)		$H_{av}$ (%)		$H_{av}$ range (%)	
	Day	Night	Day	Night	Day	Night	Day	Night
1 day	19.7	15.5	13.4	6.4	78.3	82.4	22.5	5.7
> 1 to 10 days	17.4	12.9	9.7	7.1	72.6	76.9	17.3	17.4
> 10 to 44 days	15.2	10.7	11.3	7.7	82.3	88.7	19.6	12.1
> 44 to 91 days	10.1	7.5	6.8	5.7	89.9	94.1	18.0	8.5

$T_{av}$ , average temperature;  $H_{av}$ , average humidity.

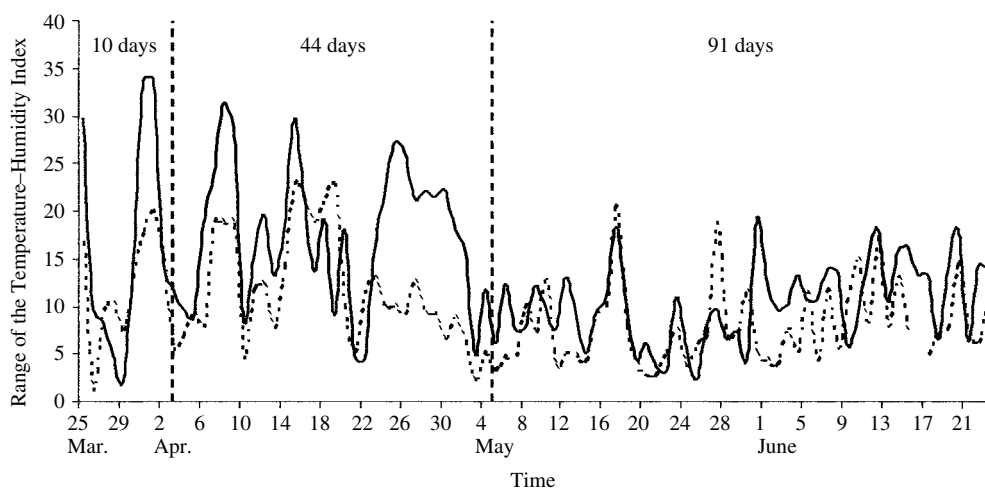


Fig. 5. Daytime (—) and night-time (---) range of the Temperature–Humidity Index for the periods of RHDV exposure to environmental conditions.

of unculturable agents include *in vitro* studies with a culturable surrogate agent that is biologically related [19], or *in vivo* studies using experimental challenge of susceptible hosts with the agent itself (as in this study). The primary concern with the use of surrogate agents is the reliability with which it is possible to extrapolate the findings across species, while the primary concern with *in vivo* studies is ethical cost. The small pilot experiments in this study were designed to verify and optimize the methods for the major study.

Our observations under natural environmental conditions indicated that virus survival was affected by duration of field exposure and the vehicle (liver or cotton) used. Virus kept dried on cotton tape, which would mimic dried excreted virus in the field, was viable for up to 10 days of exposure to environmental conditions, but not after 44 days. Although no statistical significance could be obtained for pairwise multiple comparisons for risk of death at prolonged exposure periods for cotton samples, a clear trend of reduced mortality with extended environmental

exposure was evident and considered as biologically meaningful. Failure to detect statistical significance was probably due to the small number of observations. Virus injected into bovine liver, which was used to mimic RHDV in rabbit carcasses, was still viable after 91 days. The buffer of surrounding tissue probably protected the virus from desiccation and UV light compared with the dried cotton matrix. These findings indicate that the risk of transmission of RHDV via exposure of wild rabbits to environmental reservoirs of virus is probably influenced by the nature of the source of the infecting virus. Virus in rabbit carcasses could remain infectious for at least 3 months, whereas virus secreted by infected rabbits into the environment may retain infectivity for less than half this time.

Ethical constraints did not permit the accurate determination of viral titres. However, to improve the ability to detect subtle loss of infectivity following environmental exposure, in addition to undiluted preparations, one RHDV dilution just able to produce



infection in rabbits without environmental exposure ('borderline' dilution) was included. The pilot experiments determined the probable end-points and confirmed that the proposed methodology was appropriate for conducting the larger field study.

The pilot experiments confirmed that both oral and intramuscular inoculation resulted in infection of rabbits. Cooke & Berman [20] also showed that the route of infection (intramuscular, intradermal and oral) with RHDV did not affect rabbit survival. The oral route was preferred for the longitudinal study, as ingestion of virus is considered to be the predominant direct transmission route for RHDV in the field [21], particularly in New Zealand where distribution of RHDV-covered baits is a common practice of rabbit control [22].

In the only detailed study published of RHDV survival, Smid and colleagues [10] showed that RHDV in tissue suspensions can survive at 60 °C for 2 days, and at 4 °C for 225 days. Virus from tissue suspensions dried on cotton tape was able to survive at 60 °C for 2 days and at room temperature for 1–20 days, as confirmed by 100% mortality in inoculated rabbits. However, after 50 and 150 days at room temperature, only one of two rabbits inoculated with the same virus from dried cotton tape succumbed to the disease. These observations were obtained under laboratory conditions, and conclusions for field epidemiology of RHD are limited. McColl and colleagues [11] kept rabbit carcasses at 22 °C and collected liver samples up to 30 days post-death. Samples taken up to 20 days post-death were able to infect and kill susceptible rabbits, while samples collected after 26 and 30 days did not result in mortality and only some rabbits seroconverted. Although these results were produced under laboratory conditions, they agree with our field observations that RHDV infectivity decreases over time. However, McColl and colleagues' [11] different methodology in sample processing (volume of suspension media for RHDV extraction, freezing and thawing of samples) might have affected the concentration of infectious RHDV used for inoculation and resulted in failure to kill susceptible animals after 20 days. In addition McColl and colleagues [11] retrieved liver samples from animals succumbing to RHD for inoculation, while we used liver injected with a commercial RHDV product for the experiments. In another study reverse transcriptase polymerase chain reaction techniques showed that bones from RHDV-seropositive rabbits exposed to environmental conditions contained detectable amounts of RHDV RNA

for up to 7 weeks, but the viability of virus was not tested in challenge experiments [23].

The risk of epidemics of RHDV in wild rabbit populations will be influenced by the relative proportion of susceptible individuals in the population. Field data (J. Henning, unpublished observations) suggest annual RHD outbreaks occur at the end of the rabbit breeding season. Persistence of viable virus in the environment between inter-epidemic intervals is unlikely as was shown by the loss of RHDV infectivity from cotton-tape samples and by the decline in viral infectivity from liver samples over a period of 3 months in this study. RHD outbreaks are most likely when the proportion of antibody-negative susceptible rabbits reaches a critical level and re-contamination with RHDV occurs.

Flying insects also have been strongly implicated as mechanical vectors of RHDV [24, 25]. Viral persistence in flies that feed on rabbit carcasses could provide an alternative mechanism for viral persistence in an ecosystem between consecutive epidemics. Surviving and seroconverting animals, which represented only 7% of virus-inoculated animals in this study, may also be a virus source for infection. However, a detailed study on viral persistence in recovered rabbits is yet to be conducted. The outcome of infection with RHDV was influenced by the virus concentration, which was approximated by using two dilutions of the inocula. Therefore, interpretations of RHDV infectivity and antibody responses [26] need the virus concentrations to be taken into account. Reduction of infectivity occurred between 1 and 10 days for cotton samples, and was first detected between 10 and 44 days for the liver samples.

Continuous observation of rabbits following infection allowed precise recording of incubation and survival times. Cooke & Berman [20] provided relatively imprecise estimates of survival times, because animals were observed only at 8-h intervals following exposure, and estimated survival times were based on the temperature of the cadaver and the rabbit behaviour when last seen alive. In our study, reduction in infectivity was reflected in the survival time of infected rabbits following challenge. Animals exposed to lower concentrations of virus took longer to show clinical signs and to die, suggesting that the incubation period of RHD is related to viral dose. Rabbits inoculated with samples that had been exposed to the environment for longer durations had longer incubation periods suggesting that the amount of infectious virus remaining in the sample was reduced. Similar

correlations between the length of the incubation period and the virus dose have been described for rabies virus [27] and the measles virus vaccine [28].

RHD has a very high mortality rate of 95–100% [29]. In the absence of sudden death affected rabbits deteriorate and progressively lose body weight before eventually succumbing to the disease. In most experimental RHDV inoculation studies, viral antigen was detected post-death using virus capture ELISAs on liver tissue [20] as death occurs too suddenly to allow time for blood collection for antibody detection. Intensive monitoring enabled us to bleed animals immediately before death occurred and only 8% of rabbits (three animals) had developed antibodies at the time of death. Gavier-Widen [30] was not able to detect any antibodies using the haemagglutination inhibition test on rabbit sera sampled immediately before death. She suggested that the time interval was too short to develop antibodies as all animals in this study died or were euthanized between 12 and 106 h p.i. However, the shortest interval between inoculation and death of a seropositive rabbit in our study was 98 h p.i. Plassiart and colleagues [31] noted biological signs of disseminated intravascular coagulation and early necrotic changes of liver tissue as early as 30 h p.i. Therefore, it seems possible that the pathogenesis of RHD allows, in some cases, antibody development after extremely short intervals post-inoculation. Antibody detection in survivors (and in the two other seroconverting and dying animals) was observed as early as 5 days p.i. This is the same time period that Shien and colleagues [32] observed after experimental infection. However, Shien's experiment was conducted with only two 4- to 5-week-old rabbits, which would have survived anyway because of their age-related resilience [1, 6].

As only infectious virus will result in an antibody response [33], viral replication in the body is necessary to obtain a constantly high antibody level. Antibody titres of the five surviving seroconverting rabbits ( $n=6$ ) reached their highest levels between 10 and 20 days, while Shien and colleagues [32] detected antibody peaks 3 weeks p.i. In our study, four animals retained high antibody titres until the conclusion of our study at 30 days, while one animal had declining antibody titres after 5 days and another at 20 days. It is possible that rabbits with declining antibody titres had eliminated the virus and thus had no antigenic stimulation to maintain antibody levels. Shien and colleagues [32] indicated that virus could gradually be cleared from infected rabbits, resulting in a disappearance of the

viraemia, but with virus persistence in bile and the spleen. However, none of the young rabbits in Shien's experiment (4–5 weeks old at time of inoculation) showed any antibody decline during their study.

No evidence of airborne transmission of RHDV was shown in this study as none of the uninoculated rabbits ( $n=5$ ) kept with virus-infected animals in the same room in each trial developed RHD symptoms or antibodies against RHDV. Similar observations were made by Gehrman & Kretzschmar [34], who were not able to show infection in control rabbits kept in a fly-free room at 50-cm distance from RHDV-inoculated animals.

This study was conducted in the Southern Hemisphere's autumn. Environmental conditions change with the seasons, but quantification of the impact of climate factors is difficult as they change from year to year. Climate comparisons between years and regions are most productive over longer time periods. Exposure to the UV light and fluctuations in temperature and humidity may have the largest effect on virus survival. Our study was conducted over the autumn months in 2000 and revealed changing weather conditions from a large temperature–humidity range at the beginning of the study towards a more stable, but more humid climate. Kovaliski [35] mentioned that the survival of the virus is probably shorter in the hot summer months in Australia. Higher survival rates of rabbits are found in more cool, wet regions of Australia [36] while in the lower North Island of New Zealand RHD outbreaks occur during the drier period of the year (J. Henning, unpublished data). These observations suggest that some environmental or other conditions trigger the event of an RHD outbreak and influence mortality rates. In addition to affecting viral survival, weather conditions also influence rabbit breeding, rabbit behaviour and the abundance of flying insects which are all important mechanisms for generating RHD outbreaks.

The results of this study suggest that carcasses from rabbits dying from RHDV are likely to be the reservoir for persistence of RHDV following an outbreak. Excreted RHDV from infected animals or virus coated on baits may have reduced infectivity within 1–2 weeks and probably do not persist longer than several weeks.

## ACKNOWLEDGEMENTS

This research was funded by the Foundation for Research, Science and Technology, New Zealand and the Institute of Veterinary, Animal and Biomedical

Science, Massey University, New Zealand and was supported by the German Academic Exchange Service. We thank Robin Chrystall from Himatangi and Mark Redward from Dannevirke, who gave permission for trials to be conducted on their properties. We also thank Debbie and Karen Chesterfield for husbandry of the laboratory rabbits and Daniel Russell for help with sample collection. Thanks to Laurie Sandall for her assistance with virological work and Dave Pollard for help in designing the sampling unit. We also gratefully acknowledge John Parkes and Peter Spradbrow for general advice and Mark Stevenson and Nigel Perkins for statistical advice. All experiments involving live animals were approved by the Massey University Animal Ethics Committee.

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