

# Long-term survival of New Zealand rabbit haemorrhagic disease virus RNA in wild rabbits, revealed by RT-PCR and phylogenetic analysis

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Because *Rabbit haemorrhagic disease virus* (RHDV) is highly pathogenic for rabbits, farmers illegally introduced it as a bio-control agent onto New Zealand farms in 1997. The virus was dispersed rapidly, initially causing high fatality rates in rabbits. Nevertheless, many survived and these surviving rabbits have been investigated for evidence of infection by RHDV. Livers from healthy rabbits contained RHDV-specific RNA, as shown by nested RT-PCR sequencing. The sequences of the viral capsids were related closely to the released Czech strain of RHDV, although the sequence from one rabbit was related most closely to a Spanish strain of RHDV. Phylogenetic analysis of the capsid sequences of 38 samples implied that there have been at least two introductions of the Czech virus into New Zealand, probably corresponding firstly to the original illegal introduction by farmers and secondly to the introduction of the same virus under governmental control. Genomic length sequence of two samples was obtained, suggesting that they may have retained the potential to be infectious, although this has not yet been demonstrated. The detection of genomic-length RNA in the liver of healthy rabbits suggests that even though a highly virulent virus was introduced into New Zealand, it rapidly established persistent or latent infections in a proportion of rabbits. This might account for their ability to survive in the face of virulent released virus. Moreover, the co-circulation of other strains of RHDV in the same rabbit population, such as the Spanish strain, might also impact on their susceptibility to the bio-control agent.

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## INTRODUCTION

*Rabbit haemorrhagic disease virus* (RHDV) has been demonstrated to be a highly infectious and virulent pathogen for the European rabbit (*Oryctolagus cuniculus*). It was first recognized as the causal agent of a major epidemic in domestic rabbits in China in 1984 (Liu *et al.*, 1984). The disease apparently dispersed rapidly and widely across the rest of Asia and Europe, becoming epidemic and endemic within a few years. The aetiological agent was identified as a calicivirus (Ohlinger *et al.*, 1990), a positive-sense, single-stranded RNA virus which is antigenically related to

*European brown hare syndrome virus* (Laurent *et al.*, 1997; Le Gall *et al.*, 1996; Nowotny *et al.*, 1997; Wirblich *et al.*, 1994).

The high virulence of RHDV and its rapid and efficient dispersal through the rabbit populations led to it being tested as a bio-control agent in Australia, where the rabbit population was devastating the indigenous flora and fauna (Asgari *et al.*, 1999; Sandell, 2002). In 1995, a series of field trials was initiated on Wardang Island, 5 km off the coast of Port Victoria on the Yorke Peninsula, south coast of Australia. During the course of these trials, the virus 'inadvertently' escaped from the island to mainland Australia and spread at a phenomenal rate throughout the southern states (Asgari *et al.*, 1998; Cooke *et al.*, 2000; Kovaliski, 1998). Although the local rabbit population was initially severely affected, subsequent analysis has shown that the high lethality of the virus has not been maintained, and whilst sporadic epidemics are still being recorded, they do not

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occur with the same intensity that was observed initially (Lugton, 1999).

The New Zealand Government had previously rejected the use of RHDV as a control agent; nevertheless, the virus was subsequently introduced deliberately and illegally into New Zealand in 1997 (Motha & Clark, 1998; O'Keefe *et al.*, 1998; Thompson & Clark, 1997). Many farmers introduced the virus onto their farms by baiting oats and carrots with RHDV. This was done quite arbitrarily but, in spite of this, the disease was established and the government then approved the importation, manufacture and sale of a known strain of RHDV. The nature of its introduction may have had a detrimental effect on the subsequent efficacy and dispersal of the virus. It has been suggested that many rabbits may have received sublethal doses and did not succumb to the virus, as would have been expected if the virus had been disseminated under carefully regulated conditions (O'Keefe *et al.*, 1999).

The virus that was introduced inadvertently into Australia was the Czech strain V351. The complete genome sequence of this strain has been determined (Gould *et al.*, 1997) and subsequently monitored after its escape and dispersal from Wardang Island (Asgari *et al.*, 1999). Two years after its release into Australia, the virus recovered from dead rabbits had diverged by only 2.7%. The virus subsequently introduced illegally into New Zealand was confirmed to be the same strain (O'Keefe *et al.*, 1998). However, 4 years after its introduction into New Zealand, there are many healthy rabbits in areas where the virus had been released, raising questions about the suitability of this virus as a bio-control agent (Parkes *et al.*, 2002). Recent evidence has shown the presence of RNA in the tissues of healthy rabbits from New Zealand (Zheng *et al.*, 2002). This study focuses on the phylogenetic relationships of the viruses identified in the livers of healthy rabbits.

## METHODS

**Rabbit material.** Apparently healthy rabbits were shot at eight sites in Central Otago, all in the vicinity of Alexandra, New Zealand. This area was among the first to be reported with RHDV in 1997 (Motha & Clark, 1998). All rabbits were stored at 4 °C for 24–72 h until processed; therefore, no blood samples were obtained. The liver and bone marrow of all rabbits were extracted and freeze-dried. Table 1 shows the distances between the sites and Fig. 1 shows the relative location of all sites. In view of the fact that liver samples were the only source of tissue from these rabbits, antibody status has not been determined. However, the presence of antibody from rabbits obtained under similar conditions has been published recently (Zheng *et al.*, 2002).

**RT-PCR sequencing.** Viral RNA was extracted from liver samples using the RNAgents kit from Promega. Primers for RT-PCR were designed from known sequence based on full-genome sequences (Gould *et al.*, 1997; Meyers *et al.*, 1991; Rasschaert *et al.*, 1995) and are listed in Table 2. First-strand reverse transcription to produce cDNA was performed using Superscript II reverse transcriptase (Invitrogen) with the reverse external primer for each reaction. A nested PCR was used to amplify the cDNA; first-round reactions

**Table 1.** Straight-line distance between sites (km)

	1	2	3	4	5	6	7	8	9	10
1	0									
2	39	0								
3	31	13	0							
4	35	27	33	0						
5	33	19	8	8	0					
6	42	11	20	20	13	0				
7	46	7	30	30	22	10	0			
8	42	3	29	30	21	12	5	0		
9	21	36	18	17	20	33	41	39	0	
10	9	42	33	32	33	43	49	46	16	0

(RT-PCR) utilized the external primers for the region of the genome amplified and second-round reactions (nested PCR) utilized the internal primers to produce the desired products. A total of 30 cycles of 94 °C for 40 s, 50 °C for 40 s and 72 °C for 1 min was used for both sets of primers. Both positive and negative controls were included at every stage of these reactions. PCR products were gel-purified and both strands sequenced using a PE cycle sequencing kit (Biosystems) with the appropriate primers to give approximately 500 bp of sequence for assembly and analysis. Ambiguous results were re-analysed to resolve the sequence.

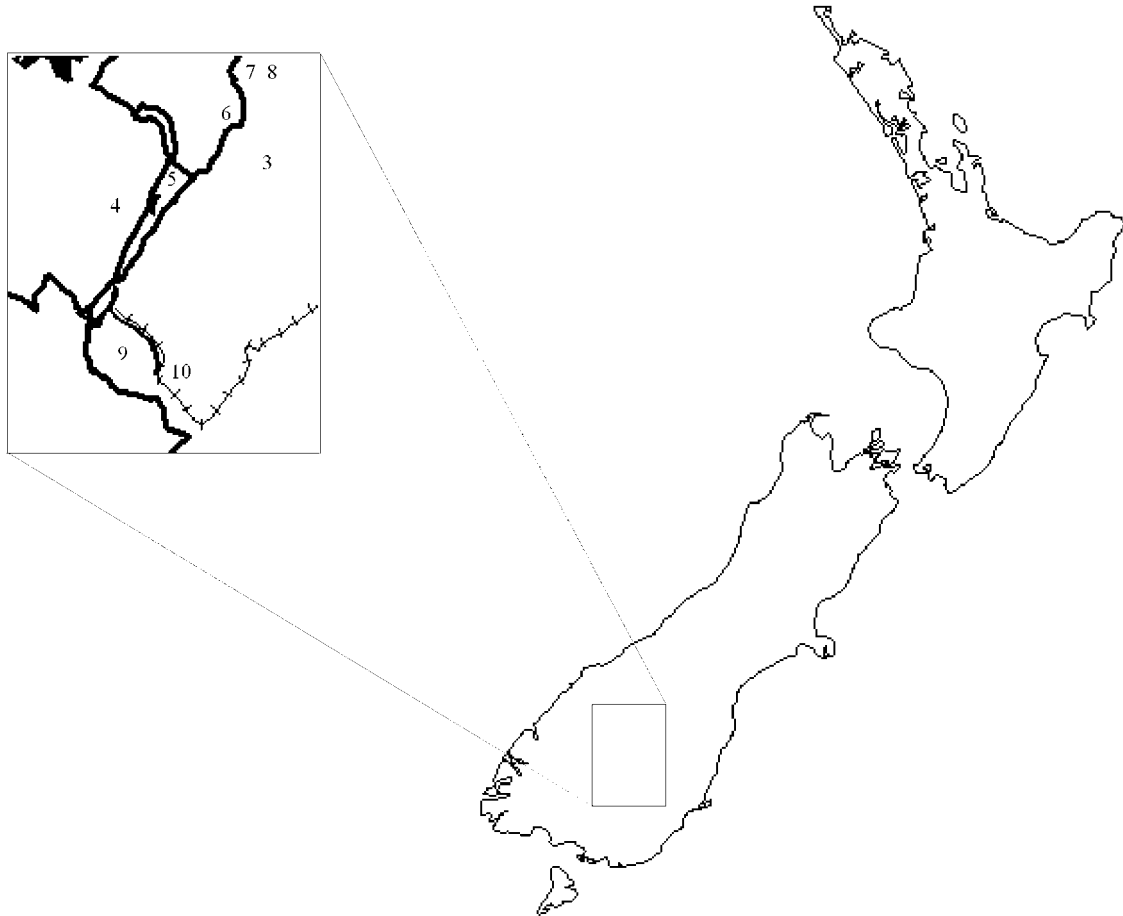
**Phylogenetic analysis.** Data produced by the sequence reactions were joined using PREGAP4 and GAP4 (Staden Package); subsequent analysis was carried out using TRANSLATE (GCG, Wisconsin Package).

The VP60 gene sequences were aligned using PILEUP (GCG, Wisconsin Package). Phylogenetic analyses were undertaken using PAUP\*, version 4.0.10b (Swofford, 2000). The optimal evolutionary model to use with each data set was estimated using MODELTEST, version 3.06 (Posada & Crandall, 1998). The optimal maximum-likelihood tree was then estimated using the K80+ $\Gamma$  model, a heuristic search and TBR branch swapping, estimating variable parameters from the data, where necessary. Neighbour-joining bootstrap support (1000 replicates) was calculated for each tree using the maximum-likelihood settings.

**Attempts to isolate infectious virus.** Domestic rabbits – supplied by Harlan – were inoculated with liver suspension that had been demonstrated to be positive by RT-PCR. Five of the freeze-dried liver samples were prepared as a clarified suspension in sterile PBS using mortar and pestles. Of each suspension, 100  $\mu$ l was injected by the intramuscular route into individual Swiss White rabbits. Rabbit sera were monitored using ELISA tests to detect the presence of antibodies and livers were examined using nested RT-PCR, as described above.

### ELISA tests

**IgG detection.** An optimized concentration of recombinant RHDV capsid protein (Marin *et al.*, 1995) was coated onto ELISA plates overnight at 4 °C in coating buffer. After washing the plates in PBS/Tween (0.1%), twofold dilutions of serum were added for 1 h at 37 °C. The plates were washed and a 1:1000 dilution of polyvalent goat anti-rabbit serum conjugated with horseradish peroxidase (Sigma) was added for 1 h at 37 °C. After washing, substrate was added and the absorption of each test was estimated. Absorption readings equivalent to at least twice the equivalent dilution of negative control serum (serum from a commercially supplied rabbit certificated as negative) were considered positive for RHDV.



**Fig. 1.** Geographical distribution of the sites from which samples were obtained in Central Otago, New Zealand.

**IgM detection.** A similar procedure was followed as with the polyvalent antibody, but instead of the polyvalent goat anti-rabbit serum, a 1:40 000 dilution of IgM goat anti-rabbit serum conjugated with horseradish peroxidase (Bethyl Laboratories) was used.

## RESULTS

### Sequencing and phylogenetic analysis of viral RNA detected in rabbit liver samples

A total of 76 liver samples obtained from eight different sites in Central Otago, New Zealand were analysed by nested RT-PCR sequencing, an approach that targeted the viral capsid gene using RHDV1 and RHDV4 for first-round PCR and RHDV2 and RHDV3 for nested PCR. The region of the capsid gene chosen was considered to be the least conserved within the capsid gene. Of the 76 liver samples analysed, 38 (50%) produced cDNA PCR products of the anticipated size (527 bp). Of the 38 positive cDNA samples, one was detected after first-round PCR; however, all the others required a nested PCR before they could be detected. Each of the PCR-positive samples was then sequenced and aligned using CLUSTAL\_X (Wisconsin Package). The maximum genetic

variability was 2.7% at the nucleotide level compared with the original Czech strain.

A phylogenetic tree was constructed using the PAUP program. The deduced maximum-likelihood tree (Fig. 2), which includes representatives of RHDV from New Zealand, Europe and China, shows that most of the positive liver samples from Central Otago produced sequences that were related closely to the Czech strain V351, which was introduced deliberately into New Zealand in 1997. However, the sequences of these samples formed two distinct groups. One group clustered close to the Czech strain introduced deliberately and the New Zealand sample sequenced in the first 2 years after release. The second group was similar to, but distinct from, the Czech strain. Very little variability was detected within each group and both groups appear to have varied equally, although they remain distinct. At five of the eight sites, we found either group 1 or group 2 viruses, but not both. From sites 3 and 10, we found viruses from both groups, and from site 6 there were group 2 viruses and a different European strain of RHDV. Within each sampling site, we cannot rule out the possibility that the rabbit population was subdivided; if this is the case, we do not

**Table 2.** List of primers used to amplify the full-length genome of RHDV

External primers were used in first-round PCR, internal primers were used in second-round PCR. Sequencing primers were used in addition to the internal primers to give approximately 500 bp of sequence for assembly and analysis. Nucleotide positions are shown in parentheses.

Region (kb)	External primers	Internal/sequencing primers	Extra sequencing primers
0–1	RHDV0001F (0001–0024) RHDV1217R (1217–1238)	RHDV0001F (0001–0024) RHDV1057R (1057–1079)	RHDV0607R (0607–0629) RHDV0382F (0382–0404)
1–2	RHDV0841F (0841–0860) RHDV2209R (2209–2232)	RHDV0868F (0868–0888) RHDV2097R (2097–2122)	RHDV1503R (1503–1523) RHDV1432F (1432–1457)
2–3	RHDV1876F (1876–1898) RHDV3098R (3098–3119)	RHDV1940F (1940–1967) RHDV3016R (3016–3041)	RHDV2515R (2515–2537) RHDV2363F (2363–2382)
3–4	RHDV2872F (2872–2891) RHDV4163R (4163–4190)	RHDV2926F (2926–2945) RHDV4127R (4127–4150)	RHDV3592R (3592–3611) RHDV3419F (3419–3437)
4–5·5	RHDV3850F (3850–3874) RHDV5621R (5621–2641)	RHDV3934F (3934–3954) RHDV5536R (5536–5555)	RHDV4634R (4634–4656) RHDV4429F (4429–4448) RHDV4981R (4981–5003) RHDV4831F (4831–4855)
5·5–6·3	RHDV2515F (5215–5238) RHDV6700R (6700–6719)	RHDV5259F (5259–5278) RHDV6234R (6234–6253)	RHDV5829R (5829–5848) RHDV5682F (5682–5698)
6–6·7	RHDV6096F (6096–6114) RHDV6774R (6774–6794)	RHDV6135F (6135–6154) RHDV6700R (6700–6719)	
6·6–7·1	RHDV6135F (6135–6154) RHDV7171R (7171–7191)	RHDV6654F (6654–6670) RHDV7023R (7023–7043)	
6·8–7·4	RHDV6663F (6663F–6685) RHDV7437R (7411–7437)	RHDV6811F (6811–6833) RHDV7437R (4711–7437)	

know from which subpopulation the samples came. As can be seen in Fig. 2, one sample (NZ51) amplified from rabbit liver (site 6) was positioned within a clade containing European viruses (Fig. 2) and is related most closely to the Spanish AST/89 strain (Boga *et al.*, 1992). Although we have worked with the capsid gene of this Spanish strain in the past, we have never introduced DNA representing sequence outside the capsid gene. As a control test, we amplified a region of the NZ51 genome outside the capsid genome (0–1 kb) to confirm that this Spanish-related sequence was not a laboratory contaminant. It is also important to emphasize that, at the time of collection of these rabbits, there were no signs of fatal epidemic RHDV.

### Sequencing of full-length RHDV genomes

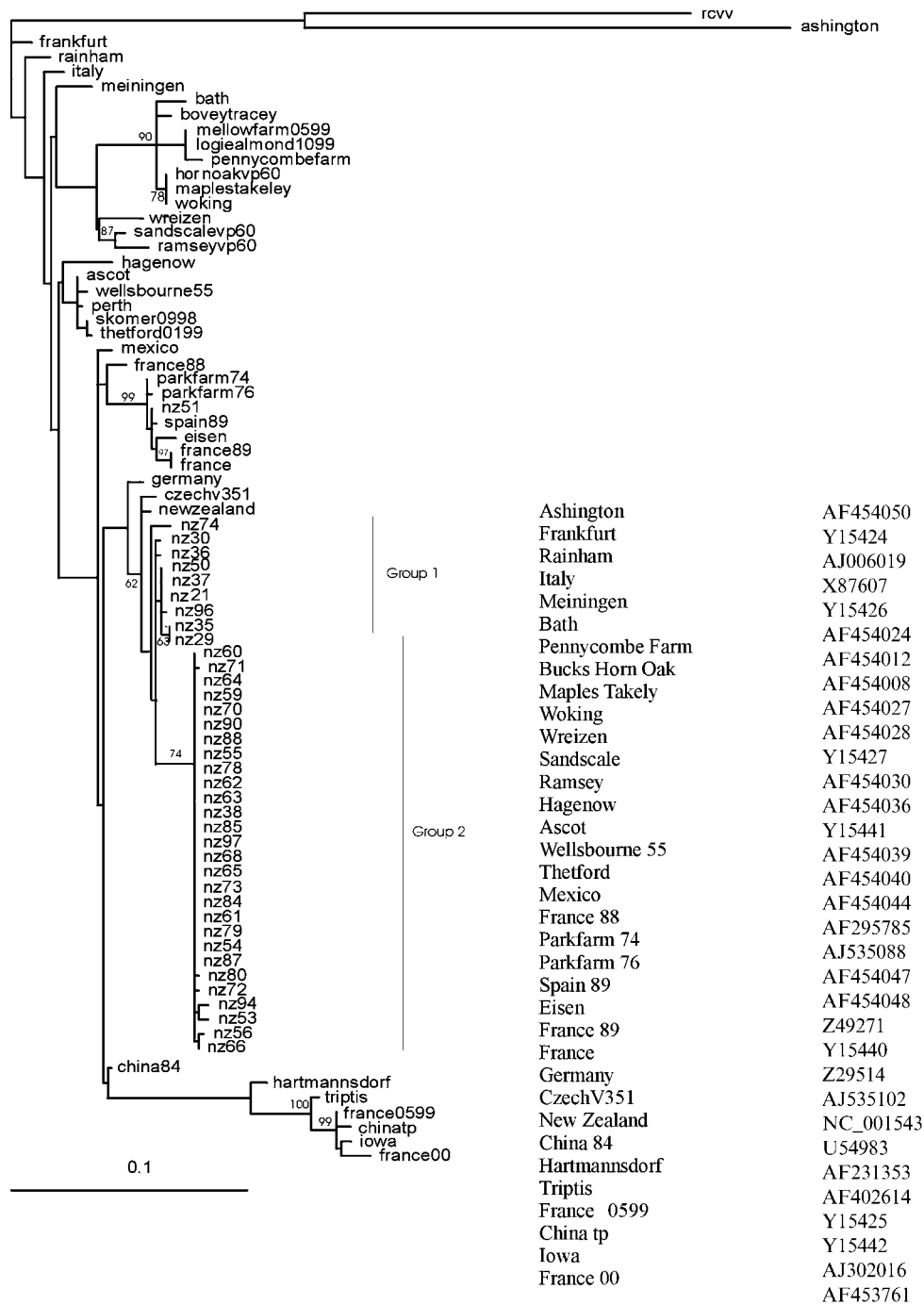
To determine if the RNA products represent full-length genomes, we obtained full-length sequences from one sample by RT-PCR as described above, utilizing all the primers defined in Table 2. These sequences were then compared against the complete genome sequence of the Czech strain. The 527 bp region and the full-length sequence of NZ54 showed 97·5 and 97·6% nucleotide identity with respect to the Czech V351 strain. The amino acid sequence was 98·6% homologous with the Czech strain. The 527 bp region that we sequenced was thought to be the most variable region of the capsid gene; however, we found that there was significant variation throughout the whole virus genome. The region from 0 to 2 kb was equally variable, i.e. to the same degree as the capsid region. The

variability within the amino acid sequence was greater than expected, suggesting that very few silent substitutions have occurred. It is worth noting that the method used to amplify the full-length genomes, i.e. nested RT-PCR using primer pairs that overlap with the previous pair, suggests that the RNA is intact, representing the complete viral genome and is, therefore, potentially infectious. It is also worth noting that 90% of a second genome has also been sequenced successfully with similar results.

### Attempts to identify infectious virus in RT-PCR-positive liver samples

In view of the fact that we had detected genomic length sequence in two rabbit livers, we attempted to isolate infectious virus from RT-PCR-positive liver suspensions. Since RHDV does not grow in cell culture, five rabbits were injected by the intramuscular route with five liver samples known to be positive. None of the rabbits showed clinical signs of infection. Sera were collected before inoculation and at the termination of the experiment. Sera were tested for the presence of RHDV-specific IgG and IgM antibody by ELISA (Moss *et al.*, 2002). There was no evidence of an immune response.

Liver suspensions were analysed by RT-PCR for the presence of viral RNA using the methods described above. No evidence of viral RNA was found in the livers of any of these animals. While these are only limited tests, the data imply that the RT-PCR-positive livers of healthy rabbits do not contain readily demonstrable quantities of infectious virus.



**Fig. 2.** Phylogenetic analysis using partial capsid sequence (see Methods) for 78 strains of RHDV. Maximum-likelihood phylogeny was calculated using PAUP\*, version 4.10b. The optimal model to use with the data (K80 + Γ) was determined using MODELTEST, version 3.06, and the variable parameters were estimated from the data. Bootstrap values (shown only on the major branches, for clarity) were estimated for this tree using the neighbour-joining algorithm under the maximum-likelihood model for 100 replicates.

## DISCUSSION

Prior to the deliberate release and dispersal of the Czech/Australian strain of RHDV into New Zealand, this country was assumed to be free of RHDV. However, on the basis of

retrospective viral antibody ELISA tests, it appeared that an RHDV-like virus had been present previously in New Zealand (O’Keefe *et al.*, 1999). Unfortunately, the identity of this virus has never been established, and since there are no reports of overt disease, it presumably circulated as a

persistent or subclinical virus infection. This is not without precedent since a non-virulent strain of RHDV in domestic rabbits in Italy has been identified and characterized (Capucci *et al.*, 1996) and RHDV-specific RNA has been detected recently in healthy rabbits sampled in the UK using the highly sensitive nested RT-PCR method (Moss *et al.*, 2002). Interestingly, we had reported that the capsid gene sequence of RNA from healthy rabbits was not readily distinguishable from the RNA detected in the liver of dead rabbits, presumed to have been lethally infected with RHDV. Serological and molecular methods also showed that a similar virus had circulated, at least in domestic rabbits for many years before overt disease was recognized (Moss *et al.*, 2002).

The consequences of the deliberate release of a supposedly highly virulent Czech strain of RHDV into New Zealand in 1997 must, therefore, be considered in the light of the evidence that there may have been a background level of immunity and/or virus-specific resistance to infection in the rabbit population. This might have influenced the variable mortality figures (10–50% mortality in the first epidemics), which resulted following the deliberate release of the virus by the farmers (Cooke, 2002).

We found RNA related closely to two supposedly virulent strains of RHDV in the livers of healthy rabbits in New Zealand, i.e. the Czech V351 strain and the Spanish 89 strain. The presence of similar viral sequences in healthy rabbits implies virus attenuation has occurred either before or, more likely, during the deliberate attempts to control the rabbit population. Alternatively, but less likely, the viruses are only virulent under defined circumstances that did not prevail in New Zealand. It is interesting to note that one liver sample produced a cDNA product from first-round PCR amplification, which indicates that there is more virus present within the liver. In our experience, only recognized lethal viruses, i.e. viruses obtained from dead or dying rabbits, have yielded PCR products on first-round PCR amplification (unpublished data). Therefore, it could be argued that this sample represents a virus related more closely to virulent RHDV. However, the fact remains that it was obtained from an apparently healthy rabbit and its sequence was not distinct from the other sequences in its subgroup. Since the livers were freeze-dried, it was not possible to confirm this by histopathological methods.

Of the 38 rabbit liver samples that contained viral RNA, all but one were related closely to the Czech strain known to have been used by the farmers who spread the virus deliberately. However, phylogenetic analysis distinguished two distinct groups. The sequences of the first group were closest to the imported Czech strain and the New Zealand strain that was sequenced in the first 3 years after the virus was introduced (O'Keefe *et al.*, 1998). The second group was related more distantly and it appeared that the divergence point of the virus occurred before the virus was introduced into New Zealand (Fig. 2). This could correspond to the virus that was imported from Australia, which would

presumably have diverged from the original Czech strain before being introduced into New Zealand. Therefore, the two groups probably correspond to the virus introduced originally by the farmers (group 2) and the second virus that was made available by the New Zealand Government for introduction (group 1). Nevertheless, since both groups of viruses were isolated from at least two sites, it is clear that they have co-circulated in the New Zealand rabbit population. Whether or not this type of interaction has influenced the epidemiology and pathogenesis of RHDV in these rabbit populations remains to be determined.

The clustering of these virus sequences around the introduced Czech strain argues strongly against the idea that they are the original New Zealand virus that is believed to have been present before 1997. However, it is possible that the unique European strain we identified at site 6 represents the pre-1997 virus. In view of the surprising nature of this observation, we considered whether or not this sequence could have been introduced inadvertently as a laboratory contaminant in Oxford when we were analysing the samples. However, although we use a recombinant baculovirus that expresses the Spanish 89 capsid gene for our ELISA test (Marin *et al.*, 1995), we have never knowingly worked with other regions of the genome of this virus in our laboratory. Therefore, as a further control, we amplified Spanish 89 sequence from outside the capsid gene of the New Zealand isolate. These experiments confirmed that the Spanish strain is present and circulating in New Zealand but at the present time we are unable to explain how it was introduced.

Since both the Czech and Spanish viruses have been recognized previously as virulent viruses, the presence of the RNA of either strain in healthy rabbits implies that even the most virulent RHDV may, in addition to causing lethal epidemics, also cause subclinical persistent and/or latent infections. It has been suggested that the unorthodox and unregulated introduction of the virus could have caused a sublethal dose of the virus to be administered to the rabbits (O'Keefe *et al.*, 1999). However, there is good experimental evidence showing that either low or high input concentrations of virus are lethal in immunologically naïve adult hosts (Teifke *et al.*, 2002). Thus, the mechanism by which the virus enters this persistent state is as yet undetermined.

The detection of RHDV-specific RNA in the liver of healthy New Zealand rabbits known to have been infected by the released Czech virus presents us with the problem of whether or not the RNA represents infectious virus? We failed to demonstrate infectivity or an immune response following inoculation of liver suspensions from known carriers of viral RNA identified by RT-PCR sequencing. However, only five samples have been tested thus far and although the RT-PCR sequencing results have been reproduced, they are not always positive in every experiment, indicating that the levels of viral RNA are extremely low. It is, therefore, possible, that the animals we injected were given insufficient RNA to demonstrate infectivity. The presence of full-genome

sequences of viral RNA indicates that the RNA may be maintained in a potentially infectious form. The area from which the rabbits were obtained showed no evidence of any RHDV epidemic at the time of collection (unpublished data). Therefore, the rabbits have either been carrying the viral RNA since the last epidemic or, as was suggested previously, there is a virus circulating that does not cause severe morbidity and mortality (Capucci *et al.*, 1998; Chasey *et al.*, 1997; Rodak *et al.*, 1990; Trout *et al.*, 1997). One possible explanation is that RHDV establishes a persistent infection in rabbits. The nature of persistence of RNA viruses is still not understood. Recent work suggests that the pathology of RHDV is caused by apoptosis (Alonso *et al.*, 1998; Jung *et al.*, 2000; Ramiro-Ibanez *et al.*, 1999). It is likely that persistence is caused by the avoidance of this pathway, so that the cells are not destroyed and the usual pathology of RHDV is prevented. Our failure to demonstrate infectivity may indicate that the virus is replicating very slowly in the rabbits. It has been suggested that apoptosis could be triggered by the accumulation of viral proteins within the cell. However, if the virus is replicating slowly then there would be only small quantities of viral proteins in the infected cells and apoptosis would probably not be triggered, thus modulating the infection by the virus. How the virus enters this persistent state has yet to be determined. However, it appears from our results that the viral RNA is able to persist in cells for a significant period of time. Perhaps in a manner similar to some flaviviruses, the virus can lie dormant in the host cells as double-stranded RNA (i.e. replicative form) associated closely with membranes to protect it from degradative enzymes (Mackenzie *et al.*, 1999; Westaway *et al.*, 1999). Whether or not this form of RHDV can be reactivated in a manner reminiscent of that described in sheep by Takamatsu *et al.* (2003), causing a virulent outbreak of *Bluetongue virus*, remains to be determined. Indeed, this could explain the first reported outbreak of RHDV when apparently healthy rabbits from Germany were introduced into China (Cooke, 2002).

Until the complete genome sequences of several of these putatively attenuated viral RNA products have been determined and compared with the assumed virulent parent viruses, it is not yet possible to identify changes in virus sequence that might determine the changes in virus phenotype. There are of course, other explanations. For example, the deliberate introduction of a virulent virus into a rapidly breeding rabbit population will inevitably result in many young rabbits being exposed to virus at a time when they are either naturally resistant to RHDV or have residual maternal antibody that could protect them against lethal infection. In either situation, these young rabbits could be exposed to released infectious virus and develop immunity that would subsequently protect them from further exposure to virulent released virus. This is entirely consistent with the detection of healthy immune domestic and wild adult rabbits in the UK – dating back to 1955 – that carry viral RNA virtually indistinguishable from the RNA of

apparently virulent virus. Further studies to investigate RHDV persistence and latency are ongoing.

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