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Molecular epidemiology of *Rabbit haemorrhagic disease virus*

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Millions of domestic and wild European rabbits (*Oryctolagus cuniculus*) have died in Europe, Asia, Australia and New Zealand during the past 17 years following infection by *Rabbit haemorrhagic disease virus* (RHDV). This highly contagious and deadly disease was first identified in China in 1984. Epidemics of RHDV then radiated across Europe until the virus apparently appeared in Britain in 1992. However, this concept of radiation of a new and virulent virus from China is not entirely consistent with serological and molecular evidence. This study shows, using RT–PCR and nucleotide sequencing of RNA obtained from the serum of healthy rabbits stored at 4 °C for nearly 50 years, that, contrary to previous opinions, RHDV circulated as an apparently avirulent virus throughout Britain more than 50 years ago and more than 30 years before the disease itself was identified. Based on molecular phylogenetic analysis of British and European RHDV sequences, it is concluded that RHDV has almost certainly circulated harmlessly in Britain and Europe for centuries rather than decades. Moreover, analysis of partial capsid sequences did not reveal significant differences between RHDV isolates that came from either healthy rabbits or animals that had died with typical haemorrhagic disease. The high stability of RHDV RNA is also demonstrated by showing that it can be amplified and sequenced from rabbit bone marrow samples collected at least 7 weeks after the animal has died.

Introduction

Rabbit haemorrhagic disease virus (RHDV) is a member of the family *Caliciviridae*, genus *Lagovirus*, and is most closely related to, but distinct from, *European brown hare syndrome virus* (Wirblich *et al*., 1994). RHDV was first identified in China in 1984 as a highly infectious disease of the domestic European rabbit *Oryctolagus cuniculus* (Lui *et al*., 1984). The disease was characterized by haemorrhagic lesions affecting, in particular, the liver and lungs, with a 60–90% mortality rate occurring 24–48 h after infection. However, rabbits less than 2 months old were unaffected by the virus. Rabbit calicivirus (RCV), a non-pathogenic variant of RHDV, was isolated in 1996 in Italy from commercially supplied rabbits. This virus induces immune protection in rabbits against challenge with the pathogenic virus (Capucci *et al*., 1996). Since 1992, rabbit haemorrhagic disease has been observed and recorded throughout the British mainland (Fuller *et al*., 1993; Trout *et al*., 1997) and in 1995 was also reported in the Republic of Ireland (Collery *et al*., 1995). Therefore it was assumed that RHDV is descended from the virus that caused a severe outbreak in China in 1984. However, antibodies to RHDV were reported in rabbit serum 12 years before the first outbreak of RHDV and were presumed to be in response to infection with an avirulent strain of the virus (Rodak *et al*., 1990). Attempts to detect RHDV by RT–PCR from sera and fixed tissue samples obtained prior to 1984 have, until now, proved unsuccessful (Nowotny *et al*., 1997; Ros Bascunana *et al*., 1997). Here we report the successful detection and sequence determination of RHDV-specific RNA in the serum, liver or bone marrow of either healthy or dead rabbits from Britain, dating back to 1955. We also present a detailed

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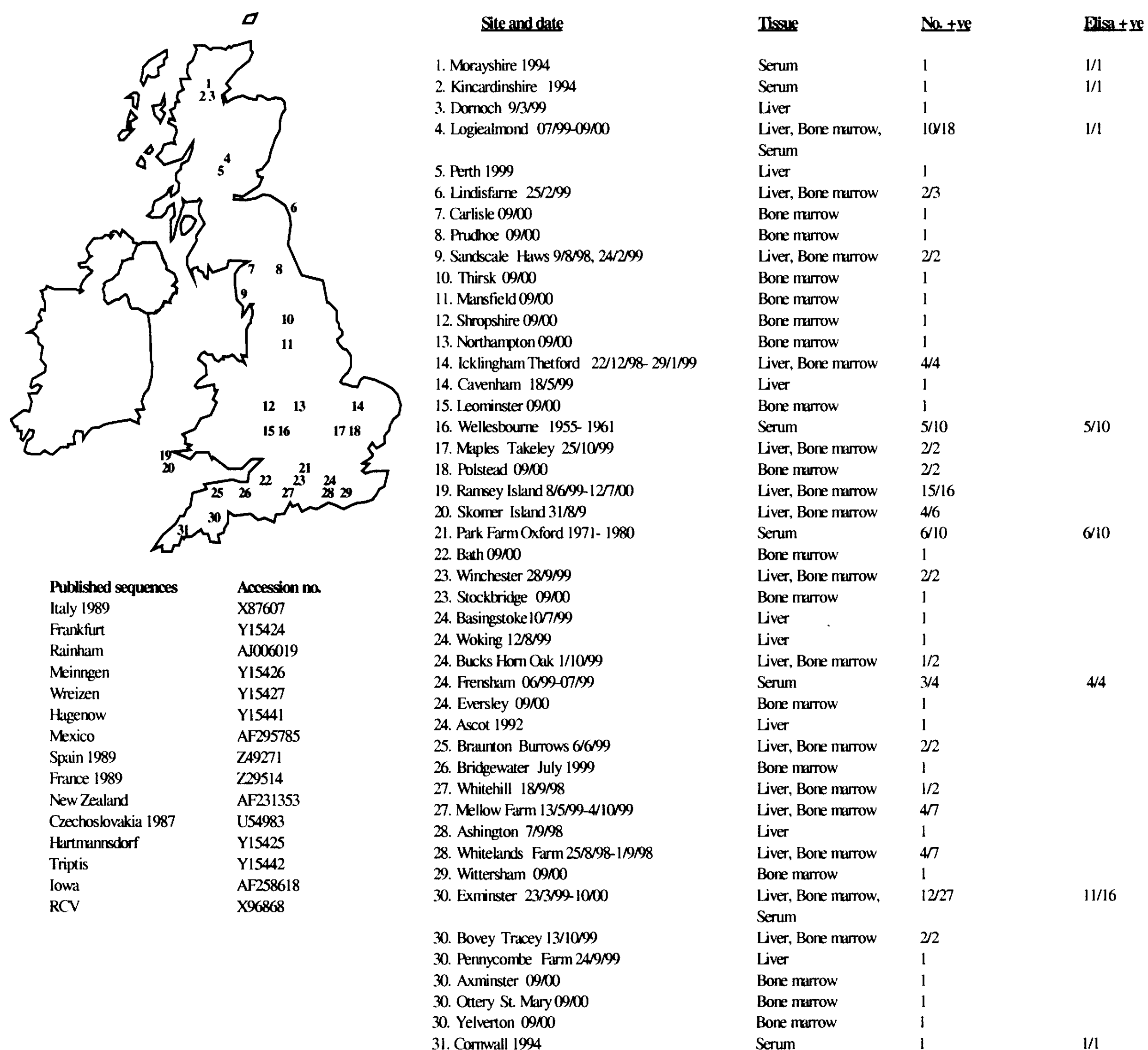
phylogenetic analysis of RHDV isolates from wide geographical regions of the Old and New World.

Methods

Rabbit material. Field samples of rabbit liver or bone marrow were collected over a period of 16 months from dead wild rabbits at field sites over a large area of Britain (Fig. 1). Liver samples from rabbits infected with the Ascot virus isolate were kindly supplied by G. Sharp at the Central Veterinary Laboratory, Weybridge, UK. In addition, three different categories of rabbit sera were collected for RT–PCR and sequence analysis. The first category comprised sera from domestic rabbits dating from 1955 to 1964, kindly supplied by N. Lyons at the Horticultural Research International (HRI), Wellesbourne, UK. The rabbits had been bred at HRI (Littlehampton, UK) from commercial stocks and the sera had been stored in 50% glycerol at 4 °C. The second category of rabbit sera, stored in our laboratory at −20 °C, originated from commercially supplied Swiss White rabbits and dated back to 1971. The third category of sera was collected from captured wild rabbits during the 1990s from a range of field sites around Britain. The rabbits were healthy when the sera were collected and stored at −20 °C. In addition to these sera from healthy rabbits, bones and liver from rabbits found dead during the 1990s were also collected for analysis. Bones from rabbits found dead during an outbreak of RHDV on Ramsey Island, UK, were also collected and then deliberately left exposed to the environment in a guarded area of a field for up to 7 weeks. At weekly intervals, one piece of bone was collected and frozen at −20 °C.

RT–PCR and sequence analysis. Viral RNA was extracted using the RNAgents kit (Promega) following the manufacturer's instructions. Primers for RT–PCR were designed from known sequences based on the



Site and date	Tissue	No. +ve	Elisa +ve
1. Morayshire 1994	Serum	1	1/1
2. Kincardinshire 1994	Serum	1	1/1
3. Dornoch 9/3/99	Liver	1	
4. Logiealmond 07/99-09/00	Liver, Bone marrow, Serum	10/18	1/1
5. Perth 1999	Liver	1	
6. Lindisfarne 25/2/99	Liver, Bone marrow	2/3	
7. Carlisle 09/00	Bone marrow	1	
8. Prudhoe 09/00	Bone marrow	1	
9. Sandscale Haws 9/8/98, 24/2/99	Liver, Bone marrow	2/2	
10. Thirsk 09/00	Bone marrow	1	
11. Mansfield 09/00	Bone marrow	1	
12. Shropshire 09/00	Bone marrow	1	
13. Northampton 09/00	Bone marrow	1	
14. Icklingham Thetford 22/12/98- 29/1/99	Liver, Bone marrow	4/4	
14. Cavenham 18/5/99	Liver	1	
15. Leominster 09/00	Bone marrow	1	
16. Wellesbourne 1955- 1961	Serum	5/10	5/10
17. Maples Takeley 25/10/99	Liver, Bone marrow	2/2	
18. Polstead 09/00	Bone marrow	2/2	
19. Ramsey Island 8/6/99-12/7/00	Liver, Bone marrow	15/16	
20. Skomer Island 31/8/9	Liver, Bone marrow	4/6	
21. Park Farm Oxford 1971- 1980	Serum	6/10	6/10
22. Bath 09/00	Bone marrow	1	
23. Winchester 28/9/99	Liver, Bone marrow	2/2	
23. Stockbridge 09/00	Bone marrow	1	
24. Basingstoke 10/7/99	Liver	1	
24. Woking 12/8/99	Liver	1	
24. Bucks Horn Oak 1/10/99	Liver, Bone marrow	1/2	
24. Frensham 06/99-07/99	Serum	3/4	4/4
24. Eversley 09/00	Bone marrow	1	
24. Ascot 1992	Liver	1	
25. Braunton Burrows 6/6/99	Liver, Bone marrow	2/2	
26. Bridgewater July 1999	Bone marrow	1	
27. Whitehill 18/9/98	Liver, Bone marrow	1/2	
27. Mellow Farm 13/5/99-4/10/99	Liver, Bone marrow	4/7	
28. Ashington 7/9/98	Liver	1	
28. Whitelands Farm 25/8/98-1/9/98	Liver, Bone marrow	4/7	
29. Wittersham 09/00	Bone marrow	1	
30. Exminster 23/3/99-10/00	Liver, Bone marrow, Serum	12/27	11/16
30. Bovey Tracey 13/10/99	Liver, Bone marrow	2/2	
30. Pennycombe Farm 24/9/99	Liver	1	
30. Axminster 09/00	Bone marrow	1	
30. Ottery St. Mary 09/00	Bone marrow	1	
30. Yelverton 09/00	Bone marrow	1	
31. Cornwall 1994	Serum	1	1/1

Published sequences	Accession no.
Italy 1989	X87607
Frankfurt	Y15424
Rainham	AJ006019
Meinngen	Y15426
Wreizen	Y15427
Hagenow	Y15441
Mexico	AF295785
Spain 1989	Z49271
France 1989	Z29514
New Zealand	AF231353
Czechoslovakia 1987	U54983
Hartmannsdorf	Y15425
Triptis	Y15442
Iowa	AF258618
RCV	X96868

Fig. 1. Geographical distribution of isolates from England, Scotland and Wales that were positive for RHDV, as assessed by RT–PCR. The map identifies the site of virus isolation, the type of tissue analysed, the number of positive samples identified at each site and the number of positive serum samples (tested by ELISA). The figure also provides the accession numbers of published sequences.

RHDV capsid protein VP60 (Boga *et al.*, 1994; Rasschaert *et al.*, 1995; Capucci *et al.*, 1996) and corresponded to nt 6096–6114 (RHDV1), nt 6135–6154 (RHDV2), nt 6700–6719 (RHDV3) and nt 6774–6794 (RHDV4). First-strand cDNA synthesis was performed using SuperScript II reverse transcriptase (Life Technologies) with the RHDV4 primer. Nested PCR was used to amplify the DNA: the first reaction (RT–PCR) utilized primers RHDV1 and RHDV4, while the second (nested PCR) utilized primers RHDV2 and RHDV3 to produce a product of 573 bp. A total of 30 cycles of 90 °C for 40 s, 55 °C for 40 s and 72 °C for 1 min was used for both sets of primers. These PCR products were gel-purified and both strands sequenced using a PE Biosystems Cycle Sequencing kit with primers RHDV2 and RHDV3 to give 527 nt of sequence for analysis.

Phylogenetic analysis. The VP60 gene sequences were aligned using PILEUP (GCG, Wisconsin Package). Phylogenetic analyses were undertaken using PAUP*, version 4.0b8 (Swofford, 2000). The optimal evolutionary model to use with each data set was estimated using MODELTEST, version 3.06 (Posada & Crandall, 1998). The optimal model ($TrNef+\Gamma$) was then used to estimate the optimal maximum-likelihood (ML) tree, estimating variable parameters from the data where necessary. Neighbour-joining (NJ) bootstrap support (1000 replicates) was calculated for each tree using the ML settings.

Antibody detection. An optimized concentration of recombinant RHDV 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 absorbance values of each plate were estimated at 492 nm. Absorbance values equivalent to at least twice the equivalent dilution of negative control serum (serum from a commercially supplied rabbit certified as RHDV-negative) were considered positive for RHDV.

Results

RT–PCR and sequence analysis

A total of 108 samples, sera ($n = 40$), liver ($n = 42$) and bone marrow ($n = 26$), from rabbits was analysed by RT–PCR and sequencing. In all experiments, known negative and positive samples were included as controls. The results in Fig. 1 show the type of sample, the year of sample collection, the geographical location of sample collection, the result of the PCR reaction for the rabbit, the ELISA result on the serum and the accession numbers of the sequences published previously. Of the 78 samples that were positive by RT–PCR, 11 were from sera collected between 1955 and 1980, i.e. up to 37 years before RHDV was first detected in Britain.

Genetic identity and phylogenetic analysis

The nucleotide sequences of the British isolates were aligned with published sequences, which included the Italian avirulent virus, RCV, and virulent RHDV isolates from Rainham (UK), the Czechoslovakian Republic, Germany, France, Italy, Spain, Mexico, New Zealand and the USA. With the exception of a strain of virus from Ashington (UK), which was 21% divergent from the other British viruses, the new isolates showed maximum nucleotide differences of 8%, indicating only limited variation amongst most of the British viruses analysed. The European isolates showed a maximum of 14% difference from the British isolates, Triptis and Braunton Burrows isolates being the most distant. Comparison with sequences from previous British isolates showed that viruses identical to the Ascot-92 virus isolate were circulating at a number of sites in Britain, and the Rainham virus isolate was most similar to the Frankfurt virus isolate. The avirulent Italian virus, RCV, was 19% different from the Ashington virus and 18% different from the other British viruses.

The ML tree for 61 viruses, rooted by RCV and Ashington virus, is shown in Fig. 2. Only viruses that have different sequences are presented in the tree. Virus sequences obtained from healthy rabbits are highlighted in red. Although the tree is monophyletic, there is a significant range of variation between the viruses in the genus. The striking genetic difference between RCV and Ashington virus with each other and also with all the other RHDV viruses implies early divergence, i.e. in the distant past and then a relatively long period of evolution before subsequent divergence to produce the more recently diverged heterogeneous viruses that currently circulate throughout Europe, central East Asia, Australia and New Zealand. For convenience, and using bootstrapping as the basis for separation of the viruses, the tree can be divided into eight major groups, six of which contain viruses from Britain. Group 1 contains two antigenically closely related but genetically distinct viruses, i.e. RCV, the avirulent Italian isolate, and Ashington virus, which was obtained from a wild rabbit within a few hours of death. Ashington virus was found to be genetically distinct (differing by up to 19% nucleotide and 18% amino acid identity) from all other viruses, including RCV, the avirulent isolate reported by Capucci *et al.* (1996). Ashington virus and RCV were used as the out-group in the phylogenetic tree. No other isolates similar to Ashington virus have been identified as yet, even from the same site. Indeed, two other isolates (not shown in Fig. 2) from this site were similar to the Ascot virus isolate. Group 2 contains the Rainham virus from Britain, together with the German and Italian viruses. Because of low bootstrapping, neither the Meiningen nor the Hagenow virus isolate has been designated a position in group 2 or any other group. Group 3 contains viruses found exclusively in Britain, the earliest isolate in this group being from the serum of a healthy commercially supplied rabbit, the serum of which had been stored at 4 °C since 1959, and the most recent isolate was from a wild dead rabbit collected in the year 2000 (not shown in Fig. 1). Therefore, virulent and apparently avirulent viruses were not readily distinguishable by phylogenetic analysis of partial capsid sequences. Despite the fact that group 3 viruses were all isolated in Britain, they were dispersed widely throughout the country. Group 4 contains isolates from wild rabbits collected at Ramsey Island and Sandscale on the west coast of Britain and Frensham in southern England; these

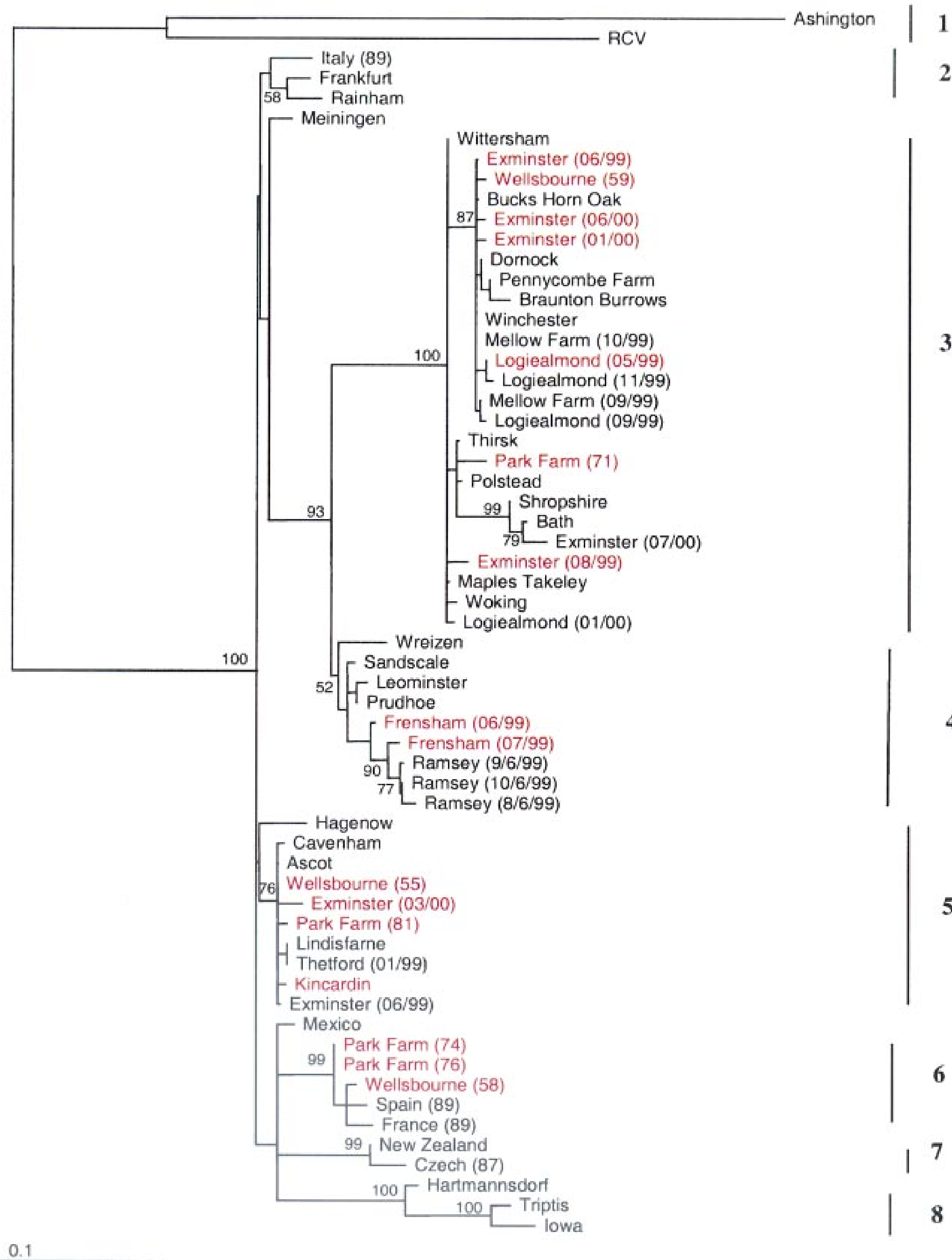


Fig. 2. Phylogenetic analysis using partial capsid sequences (see Methods) for 61 strains of RHDV. Strains of RHDV with an identical sequence to any of those shown in the phylogeny are not presented. Numbers in parentheses indicate the year of virus isolation. ML phylogeny was calculated using PAUP*, version 4.0b8. The optimal model to use with the data (TrNef+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 NJ algorithm under the ML model for 1000 replicates.

isolates were closely related to the Wreizen virus isolated in Germany in 1993. In Group 5, the earliest RHDV RNA-positive British isolate was collected in 1955 from the serum of a healthy commercially supplied rabbit kept in Littlehampton, UK. The British isolates in group 6 were from serum samples of healthy commercially supplied rabbits, collected in 1958, 1974 and 1976, and were most closely related to virulent Spanish and French wild rabbit isolates collected in 1989. Group 7 contains isolates from New Zealand and Czechoslovakia, the New Zealand virus being derived from a

Czechoslovakian isolate. Group 8 contains two German isolates and a virus that was obtained from the outbreak in the USA in 2000. The only significant geographical grouping of isolates was that seen amongst the group 3 viruses. Otherwise, there was no obvious correlation between geographical location and date of isolation for viruses in groups 3 and 5, each of which contains viruses from widely different areas of Britain. However, the British group 4 isolates were all collected from the same geographical region, west Wales and northwest England, and were similar to the Wreizen virus isolate (isolated in Germany in 1993). The group 6 British isolates were from sera obtained at a single site (Park Farm, Oxford, UK) and were similar to European isolates dating from 1989.

Examination of individual virus isolates amongst the group 3 and 5 viruses shows that there has been very little genetic variation within these groups during a period of more than 40 years. In contrast, the genetic distance between these viruses and Ashington virus and RCV in group 1 is quite extensive. Therefore, if we assume that the rates of variation are similar for these different virus groups, it follows that groups 1 and 3 must have diverged a relatively long time ago, i.e. much more than 40 years ago. It also follows that the Italian RCV strain and the British Ashington virus strain must have diverged from the remaining virus groups significantly more than 40 years ago and probably hundreds or even thousands of years ago. The tree also shows differences in the extent of divergence between groups of viruses: for example, group 3 and 5 viruses have in general diverged more than the viruses in group 4. Such results normally suggest different selective pressures on the viruses. Alternatively, they may reflect sampling bias. Another interesting observation in the phylogenetic analysis is the observation that European and British viruses from either healthy or dead domestic and wild rabbits are placed very closely together in the branching structure of the tree, indicating that there is no clear distinction between apparently avirulent and virulent viruses, at least in the region of the genome analysed. This observation was also supported when samples were obtained from rabbits that died in front of observers who immediately took field samples for analysis. There was no distinction in the sequence between these viruses and others that were collected from the sera of apparently healthy wild rabbits at the same field site. It is also worth pointing out that Ashington virus was obtained from a dead wild rabbit, which was assumed to have been killed as a result of a virulent infection that also killed $>90\%$ of the local rabbits. This implies that, although there was no obvious evidence of outbreaks due to RHDV more than 40 years ago, the genetic lineages of these virulent and avirulent viruses were present within rabbit populations.

Serum from healthy wild rabbits captured in 1999 and 2000 contains RHDV RNA

We have shown above that serum from apparently healthy commercially supplied rabbits that had been stored for more than 40 years at 4 °C contained detectable RHDV RNA, as detected by nested RT–PCR. These extraordinary results imply that some form of RHDV was circulating harmlessly throughout the commercial rabbit population in Britain during the mid 1950s, i.e. many years before the first recognized outbreak of the disease in China. We therefore decided to test, by RT–PCR and sequencing, the sera of healthy wild rabbits marked and re-captured between the years 1999 and 2000 from a variety of field sites. The captured rabbits were bled and then released for re-capture during subsequent months. Positive RT–PCR results were obtained and the sequence of the viral RNA in sera from rabbits that seroconverted during the course of the re-capture experiments was very similar to that obtained from fatally infected rabbits. Serum that was positive by RT–PCR was also recovered on subsequent re-capture of the same animals. These results show that non-immune rabbits may become infected by potentially virulent RHDV without showing any adverse effects and they then remain healthy but infected for many months.

Healthy rabbits positive for RHDV RNA also contain RHDV-specific antibodies

In separate experiments, serum samples from 10 randomly selected healthy wild adult rabbits from different regions of Britain, which had tested positive by RT–PCR, were analysed for the presence of RHDV-specific antibodies by ELISA. Each of the 10 serum samples that tested positive by RT–PCR was also positive for antibody by ELISA, whereas approximately 50% of the rabbits tested, which were negative by RT–PCR, were also negative by ELISA, implying that a high proportion of healthy immune wild adult rabbits are persistently infected with RHDV. Since these tests were performed on rabbits from field sites where known virulent infections had been reported, it can be assumed either that the healthy rabbits were infected with a potentially virulent virus or that two viruses which have a very similar sequence but significantly different virulence for rabbits are co-circulating in the same rabbit population.

Long-term survival of viral RNA exposed in the natural environment

During the course of these studies, we had demonstrated that the marrow obtained from the bones of dead rabbits collected at various field sites throughout Britain was a reliable source of RHDV RNA. We therefore decided to examine the stability of viral RNA in rabbit bone marrow that had been left exposed to the environment. During June (1999), bones from known RHDV-positive wild rabbits, collected on Ramsey Island, UK, were placed in a protected area of a field and left exposed to the environment. Each week, a tibia or femur containing marrow was taken and analysed by RT–PCR and sequencing for the presence of RHDV RNA using the methods described above. Positive results were obtained during each of the first 7 weeks after exposure of the bones. The sequence of

the virus from each consecutive sample was identical and corresponds to the Ramsey Island virus seen in Fig. 2. These results demonstrate that RHDV RNA in the marrow of exposed rabbit bones can survive for extended periods in the environment, thus increasing the opportunity for the virus to be spread by animals and birds carrying the bones.

Discussion

Rabbit haemorrhagic disease was not recognized prior to 1984 when a major epidemic occurred in China (Lui *et al*., 1984). Subsequently, antibodies specific for RHDV in rabbit sera, dating from before 1984, confirmed the presence of the virus in southern Europe (Rodak *et al*., 1990). It therefore seemed logical to assume that RHDV had been introduced into China and, since rabbits from Germany were known to have been imported into China immediately before the epidemic outbreak, this seemed to be the most likely source of the virus. Nevertheless, the evidence for RHDV in rabbits in Europe prior to 1984 was only circumstantial. According to previous reports (Nowotny *et al*., 1997; Ros Bascunana *et al*., 1997), attempts to detect RHDV RNA in rabbit sera had proved unsuccessful. Using nested RT–PCR, we detected RHDV RNA in sera dating back to 1955 from healthy commercially supplied rabbits and also in the stored sera of apparently healthy wild rabbits, collected in different regions of Britain during the 1990s. In all cases, the sera of the rabbits that were RHDV-positive by RT–PCR contained antibodies against RHDV. At this stage of our investigation, it has not proved possible to demonstrate that the RNA from the commercially supplied sera in long-term storage retains infectivity for rabbits. This may reflect the limited quantity of stored serum available for use as a challenge dose in antibody-negative commercially supplied rabbits. The RNA in the stored sera is genetically closely similar to virus strains circulating today, strains that are presumed to be virulent for rabbits. This is further supported by the fact that the sequence of RHDV RNA obtained from healthy wild rabbits was, in some cases, identical to that of RNA detected in the bone marrow of rabbits collected from the same field sites and believed to have died with clinical and pathological signs of virulent infection. However, it has to be admitted that the sequencing work has focused on partial capsid gene sequences. Whole genome sequencing has not yet been carried out with these viruses; this is a long-term objective.

Another implication of our results is that RHDV was circulating in Britain, presumably in a relatively innocuous form at least 30 and, on the basis of the phylogenetic tree, probably many more, years before the first recorded RHDV outbreak in China in 1984. Indeed, viruses isolated in Europe, China and Britain have very similar sequences and it is therefore highly likely that RHDV was also circulating throughout Europe at or before this time. Moreover, the intra-clade divergence within groups 3 and 5 represents variation that occurred over 40–50 years and many of the lineages have been diverging for much longer than this, i.e. perhaps for hundreds of years. Therefore, since the group 1 viruses clearly show significantly more genetic variation, they must have been diverging for a far longer period of time, i.e. possibly for thousands of years. If we examine the topology of the tree, there was a long time-period between the divergence of group 1 viruses and viruses in groups 2–8, during which period there were no new lineages isolated, possibly reflecting rabbit population crashes due either to myxomatosis or to other environmental factors. The more intense period of cladogenesis that occurred recently has produced a series of distinct lineages comprising groups 2–8. This apparently higher rate of variation possibly reflects intense breeding or farming practices, the mobility of domestic rabbits used for meat or as pets, the recovery of wild rabbit populations from the myxomatosis-induced crashes of the 1950s and the capture in one country and subsequent release in other countries, such as Spain and France; this has occurred in recent times. During the past century, commercial movement of rabbits has rapidly accelerated and this shows in the wide range of geographical dispersal of closely related strains within individual genotypes. It is therefore surprising that group 3 in the tree contains only British viruses. However, this could simply reflect the limited range of samples from Europe that have been analysed at this time.

The interpretation of our results can be summarized as follows: (i) RHDV has circulated in Britain and presumably throughout Europe for at least 50 years but probably for a significantly longer period of time; (ii) the sequence data imply that although RHDV is normally associated with highly virulent epidemic outbreaks, it probably circulated harmlessly throughout Europe and possibly parts of Africa and Asia for many centuries or even millennia prior to emerging as a highly pathogenic virus that decimated rabbit populations in Asia and Europe. The factors that determined the apparent alteration in phenotype have not been identified yet; (iii) we have tentatively identified eight phylogenetic groups of RHDV on the basis of serological and sequence data representing part of the VP60 capsid gene demonstrating significant genetic heterogeneity among these viruses; (iv) there is relatively little obvious correlation between individual viruses isolated from particular geographical regions, indicating that they are dispersed very efficiently, probably aided and abetted by human commercial activities and passive transfer by avian and insect species; (v) the re-capture experiments indicate that a significant proportion of rabbits older than 6 weeks with no detectable RHDV-specific antibodies may become infected by RHDV without developing clinical signs of infection; (vi) despite the reported high virulence of RHDV for rabbits, the evidence suggests that virulent viruses or genetically very similar avirulent viruses are capable of persisting in rabbits in the presence of antibody without causing overt disease, since genetic differences between virulent and avirulent RHDV are

not immediately apparent from the sequence data obtained thus far; (vii) groups of RHDV strains appear to have evolved to different extents, probably reflecting the different selective pressures to which they have been exposed; (viii) RHDV RNA survives for extended periods of time in the marrow of rabbit bones exposed to field conditions. This form of long-term stability of viral RNA may contribute significantly to the success with which the virus spreads and persists in the environment.

Clearly, a long-term series of experiments will need to be performed *in vivo* before many of the above suppositions can be confirmed but the data suggest that the virulence of RHDV for rabbits may not be defined simply by unique genetic determinants in the virus. However, until complete genome sequences have been determined for a significant number of virulent and apparently avirulent viruses, it will not be possible to test this hypothesis.

It is perhaps significant that another pathogenic virus for rabbits, i.e. myxoma virus, was first reported in an epidemic form in Britain in the 1950s. As far as we are aware, the impact of this virus on RHDV epidemiology has not been assessed and, to date, there have been no significant comparative molecular or serological studies between myxoma virus and RHDV in rabbit populations. Whether or not death rates due to RHDV could be influenced by the presence of myxoma virus in the same animals needs to be assessed. This type of analysis will form the basis of our future research.

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