Molecular Epidemiology of *Rabbit haemorrhagic disease virus*

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by

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Abstract

European rabbits were first domesticated in Roman times but wild rabbits are pests due to the enormous damage they do to farmlands. The introduction of Myxomatosis in the 1950's initially reduced rabbit numbers, but they rapidly evolved genetic resistance to Myxoma virus (MYXV). Since Rabbit haemorrhagic disease virus (RHDV) is a severe pathogen of rabbits, it was released as a second rabbit bio-control agent in Australia and New Zealand. However, its effectiveness as a bio-control agent is now being questioned.

This thesis uses molecular epidemiology to describe the origins and evolution of RHDV. Liver from healthy rabbits was examined for the presence of RNA. Full-length genomes were detected and sequenced. A database of full-length RHDV genomes was compiled from healthy rabbits and rabbits presumed to have died of RHDV. Detailed analysis revealed relatively high frequency recombination amongst these viruses.

The presence of RHDV genomes in healthy rabbits suggests that RHDV can circulate harmlessly. This has significant implications for the use of RHDV as a bio-control agent and could explain the presence of antibodies in rabbit populations prior to the first recognised epidemic in China in 1984. Phylogenetic analyses performed in this thesis demonstrate that this epidemic was independent of others in Europe, implying that RHDV can readily switch between virulent and avirulent states. Although recombination may partly explain theses switches in virulence, other mechanisms such as mutagenesis were also considered.

A strain of RHDV that has circulated in the absence of disease for decades was identified in healthy rabbits on Lambay Island. This is the first identification of a persistently circulating virus in wild rabbits.

The possibility that RHDV integrates into the DNA of rabbits as a survival strategy was investigated, preliminary results are encouraging and will be reported.

Evidence of interactions between RHDV and MYXV was sought using antibody seroprevalence to indicate circulating virus. There was no evidence of MYXV influencing RHDV epidemiology or *vice versa*.

In conclusion, despite initial evidence to the contrary, RHDV naturally circulates as a non-pathogenic virus; the implications for the origin and its continued use as a bio-control agent are discussed.

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List of Abbreviations

BLAST	basic local alignment search tool
C protein	capsid protein
CFAV	Cell Fusing Agent virus
CLP's	core-like particles
CSA	Cell Silent Agent
DIC	disseminated intravascular coagulation
DNA	deoxyribonucleic acid
dsRNA	double stranded RNA
EBHSV	European brown hare syndrome virus
ELISA	Enzyme-linked immunosorbent assay
EtBr	Ethidium Bromide
FBS	fetal bovine serum
GMO	genetically modified organism
gRNA	genomic RNA
HA	haemagglutinin
HAI	haemagglutinin inhibition
HCV	Hepatitis C virus
hpi	hours post infection
HSP	high-scoring segment pairs
IRES	Internal ribosomal entry site
KRV	Kamiti River virus
MAFF	Ministry of Agriculture Fisheries and Food (now DEFRA)
MHV	Murine Hepatitis virus
ML	maximum likelihood

MP	maximum parsimony
MYXV	Myxoma Virus
N-J	neighbour-joining
ORF	open reading frame
PCR	polymerase chain reaction
pi	post infection
RC	replicative complexes
RCV	Rabbit calicivirus
RdRp	RNA-dependent RNA-polymerase
RF	replicative forms
RHDV	Rabbit haemorrhagic disease virus
RHD	Rabbit haemorrhagic disease
RI	replicative intermediates
RNA	ribonucleic acid
RT	reverse transcriptase
RT-PCR	reverse-transcriptase polymerase chain reaction
sgRNA	sub-genomic RNA
sRHDV	smooth RHDV
UTR	untranslated region
VEEV	Venezuelan Equine Encephalitis virus
WEEV	Western Equine Encephalitis virus

Chapter 1

Introduction

1.1 The European Rabbit

The European rabbit (*Oryctolagus cuniculus*) is one of the most successful colonizing animals known. From its obscure beginnings in the southern Iberian peninsula (Corbet, 1994, Zeuner & Sutcliffe, 1964), it has spread across the world, and is now present on every continent except Antarctica having colonized 800 islands worldwide (Flux, 1994). The rabbit is a member of one of 40 extant species of the family *Leporidae.* This includes the *Lepus* species (hares) and the *Sylvilagus* species (American cottontails). The closest relatives to the European rabbit are the *Sylvilagus* spp. of America and *Poelagus marjorita*, which is found in Africa (Corbet, 1994). A distinguishing feature of the European rabbit is its distinctive social and burrowing behaviour (Corbet, 1983).

The fossil record shows that identifiable *O. cuniculus* remains were present on the Iberian peninsula by the late and middle Pleistocene (Corbet, 1994). It has been assumed that the European rabbit arose in the western Mediterranean basin, i.e. both south Iberia and north-western Africa. Recent evidence has revealed that the fossil record in North Africa shows an absence of *O. cuniculus* during the Pleistocene period. However, it should be noted that lack of evidence for the presence of rabbits may be due to gaps in the fossil record. However, the first identifiable remains occur during the Neolithic period suggesting that the presence of the rabbit in northern Africa might be due to introduction by humans. By 2-300,000 years ago the rabbit had reached Southern France and Britain (Corbet, 1994, Sutcliffe, 1964), whether this is due to migration by natural means or because of human influences remains uncertain. In the UK the rabbits did not survive the Ice age (110,000-10,000 years ago), and are only found there again in the 12th Century as a result of the Norman invasion of 1066. Subsequently the rabbits spread

northwards, reaching Northern Scotland in 1793, 500 years after being introduced into Britain (Barratt-Hamilton, 1912).

The first recorded domestication of the rabbit was during the Roman Empire (Zeuner, 1963), and by the 12th Century the rabbit warren was an integral part of European village life. In Britain rabbits were firmly established as part of the food chain by the 12th Century (Sheail, 1971, Sheail, 1978, Sheail, 1991, Veale, 1957). It is from these domesticated rabbits that the populations of wild rabbits in much of Northern Europe are descended. In Germany it took three hundred years for wild rabbit populations to become established after the introduction of domesticated rabbits.

Genetically most of the European wild rabbit population is more closely related to the domestic rabbit than the original smaller Iberian population, although they have reverted to the wild type colour. Fitter (1959) suggested that the only true wild rabbits were to be found in the Iberian peninsula and some Atlantic islands, and that all other wild rabbits were remnants of domesticated stock accounting for their larger size. Although this is an intriguing theory, it is probably not a true explanation for body size. In Europe rabbit weight decreases clinally from north to south, and this is reflected in the size of the animals, their reproductivity and the distribution of the burrows (Rogers *et al.*, 1994). Rabbits in Iberia are smaller, grow faster, have smaller litters, breed younger and die sooner than their larger northern cousins. This difference in size is also seen in Australia (Myers *et al.*, 1994), where the differences are due to the climate, with the central arid/dry and coastal wetter areas inducing a similar cline to that found in Europe, and the rabbits found in the wetter areas being equivalent to those in which the northern European rabbits are found. In summary most of these differences are due to the need of the rabbit to

adapt to its environment. In the semi-arid regions of Australia this is a hot, dry, sodium-rich, and food-poor environment (Blair-West *et al.*, 1968).

The difference in climate also affects the reproductive season, in Spain the females become gravid at 3-4 months (Rogers *et al.*, 1994), whilst the males are able to be reproductively active at 4-5 months, and the breeding season lasts from December to March. In Southern France, the females become gravid at 6 months, and are on average 10-15% heavier than their Spanish cousins. The males also mature later, at 6 months, and the breeding season is from early January to the end of May. In northern Europe the breeding season is even later, running from early march to mid-August/mid-September, although in regions where the climate is mild reproduction can occur during other months (Brambell, 1944). Both the male and female reproductive cycles can be predicted from a combination of the climate, food availability and food quality (Soriguer & Myers, 1986). Interestingly the climate has an impact on male reproduction, whereas female reproduction is more affected by food availability and quality.

In addition to the factors discussed above rabbits are affected by a variety of pathogens, these include parasites and microorganisms such as coccidiosis (*Eimeria steridae*), pseudo-tuberculosis (*Pasteurella pseudotuberculosis*) and rabbit syphilis (*Treponema cuniculi*) (Stephens, 1952, Thompson & Worden, 1956). Rabbits are also infested by helminth parasites (Boag, 1989). However, much of the work on rabbit pathogens has focused on the Myxoma virus (MYXV), which causes myxomatosis, (discussed later), and Rabbit haemorrhagic disease virus (RHDV) which is discussed below.

1.2 Rabbit haemorrhagic disease virus

1.2.1 Virus classification

Rabbit haemorrhagic disease virus causes a fatal disease when it infects European rabbits (*Oryctolagus cuniculus*). This disease was first described in China in 1984 (Liu *et al.*, 1984). Originally RHDV was considered to be either a parvovirus or a picornavirus. However, it was finally classified as a calicivirus in 1990 (Ohlinger *et al.*, 1990, Ohlinger & Thiel, 1991, Parra & Prieto, 1990). Caliciviruses are so called because of the cup-like depressions on the capsid surface (Calix means cup in Latin). The *Caliciviridae* are sub-divided into four groups, RHDV shares the genus *Lagovirus* with the antigenically related European Brown Hare Syndrome Virus (EBHSV) (Thiel & Konig, 1999). Rabbit haemorrhagic disease virus is an icosahedral, non-enveloped positive-sense single-stranded RNA virus with a genome length of 7.5 KB. The virus is 35nm in diameter and when viewed by negative staining electron microscopy has the typical cup shaped depressions on its surface associated with viruses in the family Caliciviridae. Like most other caliciviruses, it is not yet possible to reproduce the virus in cell culture (Meyers *et al.*, 1991a, Ohlinger *et al.*, 1990, Parra & Prieto, 1990, Wirblich *et al.*, 1996).

1.2.2 RHDV Genome Organisation

Two genomic RNA species are found in RHDV, a genomic RNA (gRNA) of 7.5kb and a subgenomic RNA (sgRNA) of 2.2kb. The two species are co-linear for the last third of the RNA and both are covalently linked to a genome-linked protein (VPg) (Meyers et al., 1991a) and are encapsulated. These RNA species are found in equimolar amounts in the livers of rabbits infected with RHDV (Boga et al., 1999).

Although truncated the sgRNA is most likely derived from the gRNA, as its nucleotide sequence always corresponds with the equivalent sequence in the gRNA. If the sgRNA were to originate from the sgRNA already present within the capsid protein, then we would expect some degree of genetic separation between the two genetic species. Therefore although the sgRNA is capable of self-replication, it appears that this is not a mechanism employed by RHDV (Morales *et al.*, 2004).

1.2.3 Polyprocessing of RHDV polyprotein

The 7.5kb gRNA encodes two open reading frames (ORF), ORF1 encodes a 257kDa polyprotein, which is cleaved into nine products (Boga *et al.*, 1999, Meyers *et al.*, 2000). The complete processing of the polyprotein and the mechanism by which cleavage occurs has not yet been fully elucidated, in part, because the virus cannot be propagated easily in cell culture. Therefore, in order to determine the products of the polyprotein, partial constructs of the entire genome must be inserted into a plasmid and amplified either in *Escherichia coli*, or in a recombinant virus such as vaccinia virus.

Initially the virus protein was expressed in *E.coli* with several overlapping plasmid constructs to determine the mechanism by which the polyprocessing occurred (Wirblich *et al.*, 1996). This produced a genetic map of eight polypetides:

NH_2 – p16 – p23 – p37 – p41 – p15 – p15 – p58 – p60 – COOH

Previous work had determined that this was a two step process (Martin Alonso *et al.*, 1996) and the genetic map above is a result of both steps being completed.

In further work cultured hepatocytes infected with RHDV were used (Konig *et al.*, 1998). The drawback of this system is that it is extremely difficult to use. Nevertheless the post-translational modification was shown to be a two-step process

as inferred from the *E.coli* system. Konig *et al.* (1998) showed a third step in which the p41 protein was cleaved producing an additional two or three products. The polyprocessing strategy as determined using cultured hepatocytes is shown in Figure 1.1.

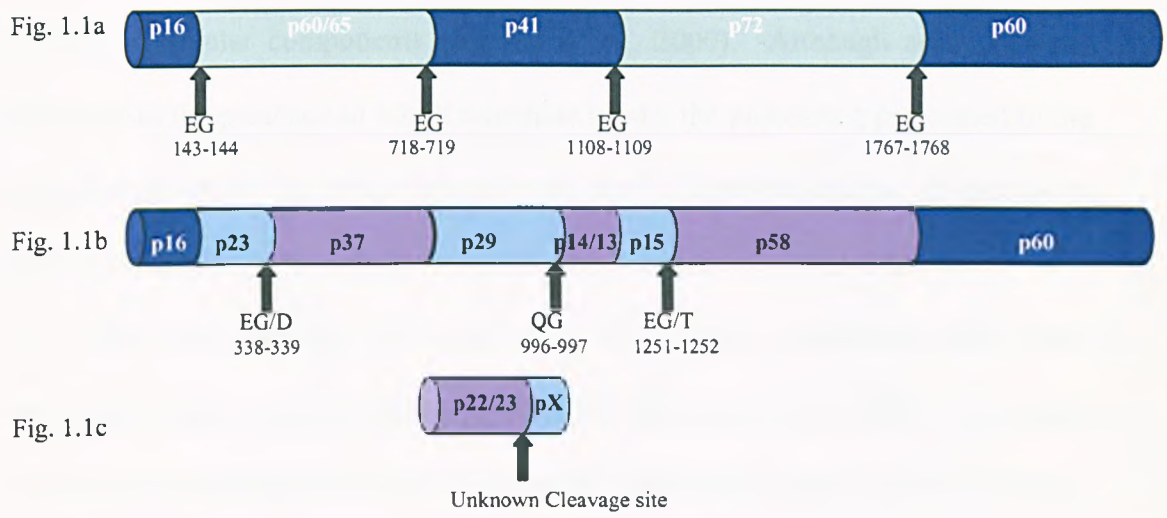

Figure 1.1 The polyprocessing of RHDV ORF1 as described by König *et al* (1998). The three rounds of processing are shown as parts a, b and c. The cleavage points are as described by Joubert *et al* (2000).

The first protein, p16, is thought to be highly unstable as very little of this protein was recovered, either in the vaccinia system or in the hepatocyte system (Konig *et al.*, 1998, Meyers *et al.*, 2000). The cleavage of p29 into p22/23 and pX is as yet undefined. Protein p29 contains an extremely hydrophobic region at its carboxyterminus whilst the rest of the protein is moderately hydrophilic. This explains why the order is assumed to be p22/23 and then pX. However, the effect that this has upon processing has yet to be determined.

The production of the polyprotein in a vaccinia system gives definition to pX (Meyers *et al.*, 2000), under this system the proteins produced are:

NH_2 – p16 – p23 – p37 – p23/22 – p5 – p13 – p15 – p58 – p60 – COOH

(where p5 corresponds to pX (Fig. 1.1)).

The differences seen between all three systems are relatively small and are most likely an artefact of the methods used. It is thought that the differences found in the cultured hepatocyte system (Fig. 1.1) are due to post-translation modification by viral or cellular components (Meyers *et al.*, 2000). Although analyses were performed in the presence of rabbit reticulate lysate, the processing performed in the whole cell system will give the most accurate result. Thus the scheme represented in Fig. 1.1 is most likely to correlate with the processing seen in the rabbit.

The nine cleavage sites (see Fig. 1.1) of the polyprotein have been determined, these are also found in EBHSV (Meyers *et al.*, 2000). The most common site for protease activity is a Glu-Gly (EG) site. There are 10 EG sites in the RHDV genome (Joubert *et al.*, 2000), of these, 4 have highly efficient proteolytic activity and these give rise to the first round of polyprocessing (Fig. 1.1a). Of the remaining EG cleavage sites, four have not been shown to be cleaved and two show only weak proteolysis. These two are in some way responsible for the second round of polyprocessing, although mutation studies have shown that both Glu-Asp (ED) and Glu-Thr (ET) mutations can result in cleavage (Fig. 1.1b). It is thought that these cleavage sites may require viral or cellular factors to increase the efficiency of cleavage. The final known cleavage site is a Gln-Gly (QG), this p41 cleaves to produce polypeptides p29 and p14/13 (Meyers *et al.*, 2000). The effectiveness of the cleavage sites is determined by the amino acid sequence immediately before and after the EG. The most common sequence is NVMEGKAR, if the Methionine (M)

is changed to a Serine (S) then there is a decrease in cleavage activity. If the N residue (Asparagine) is changed to a more basic residue this also hampers cleavage activity.

The final site that produces p22/23 and p5 from p29 does not appear to be dependent on the 3C protease. Both sites which process p41 are completely dependent on the site EG (718-719) for processing. However, as yet no mechanism by which p29 is processed has been elucidated. Picornaviruses, which are analogous in their polyprocessing to RHDV, use a second protease, a 2A protease (Cohen *et al.*, 1996), to complete the processing of the polyprotein. A second protease may be responsible, but no part of the RHDV genome corresponding to the 2A protease motifs has been identified. The processing of cardioviruses and apthoviruses contains a mechanism known as spontaneous hydrolysis, where the bond between the amino acids is weakened. The proteins are then cleaved without assistance (Hahn & Palmanberg, 1996, Hahn & Palmanberg, 2001, Ryan & Drew, 1994, Ryan *et al.*, 1991). In the RHDV genome no sequence homology with these viruses has been demonstrated, and whilst it cannot be ruled out entirely, it is unlikely that self-processing does occur (Thumfart & Meyers, 2002). Finally, the processing of p29 may be accomplished by other viral and cellular factors, which as yet have not been identified.

Of the nine proteins that are derived from the polyprotein, the functions of five have been elucidated. One is a structural protein, the VP60 (p60), which comprises the capsid protein, and because of its direct exposure to the host immune system is subjected to the strongest evolutionary selection pressures. The other four are non-structural proteins: a helicase (p37), so named because of its sequence similarity to a superfamily of virus-encoded NTP-helicases (Boga *et al.*, 1999), the

VPg (p14/13) is a viral cap which is covalently bound to the 5'-end of both the gRNA and sgRNA, the residue responsible for this binding is the Tyr 21 (Machin *et al.*, 2001). This is thought to initiate translation due to the small size of the untranslated regions (UTR's) of RHDV (Meyers, 2003). The uridylation of the VPg is catalysed by the polymerase, under experimental conditions the RNA template is not required, but under some conditions the presence of the template may prove vital (Machin *et al.*, 2001). The 3C protease (p15) is a trypsin-like serine protease, which is very similar to that found in picornaviruses (Boniotti *et al.*, 1994). The three constituents of the 3C protease are His 1135, Asp 1152 and Cys 1212, of which Cys 1212 is the nucleophillic residue, His 1135 is analogous to His 40 of the picornavirus 3C protease and Asp 1152 is the acidic residue analogous to His 20 of the picornavirus 2A protease. Interestingly the positioning of the residues is closer to that of the picornavirus 2A protease, but the size of the protease is closer to that of the 3C protease. This is the protease responsible for the processing of the polyprotein, which releases the non-structural proteins during a proteolytic cascade (Martin Alonso *et al.*, 1996). Finally the last non-structural protein is the RNA-dependent RNA polymerase (RdRp) (p58). This is the major enzyme involved in replication of the RHDV genome (Lopez Vazquez *et al.*, 2001). Under experimental conditions the 3' terminus of the template RNA is held by a recombinant enzyme, and loops back on itself. The RHDV RdRp initiates at the free 3' OH and extends towards the 5' end of the molecule, resulting in a hairpin like RHDV product. There is also the possibility that two identical RNA molecules may participate in a bimolecular mechanism as occurs in polioviruses (Hey *et al.*, 1986, Lubinski *et al.*, 1987, Neufeld *et al.*, 1991, Plotch *et al.*, 1989).

ORF2 is frameshifted with respect to ORF1, and encodes a 10kDa protein (VP10) (Boga *et al.*, 1999), that is thought to be a minor component of RHDV virions, because the packaging of the virions can occur in the absence of VP10. The start codon is located upstream of the termination codon of ORF1, with the nucleotide position -3 -5 being the most likely candidate. The termination codon for ORF1 has a significant effect on translation of ORF2 and any changes may result in mistranslation (Meyers, 2003). The mechanism of translation initiation is as yet undetermined, there are three possible theories, firstly that the ORF2 utilises an Internal Ribosomal Entry Site (IRES), that allows it to start translation in the absence of the AUG. Secondly, that a frameshift occurs inside the ribosome producing the ORF2 protein as a fusion protein with the ORF1. Thirdly there is reinitiation of translation. However the fact that 84 nucleotides are needed for translation and that the AUG is unnecessary for translation initiation suggests that none of these theories is correct, and a new mechanism needs to be determined (Meyers, 2003).

There are two untranslated coding regions (UTR), one at the 5' end consisting of 9 nucleotides and one at the 3' end consisting of 59 nucleotides (Gould *et al.*, 1997). The 5' end is protein-linked to a VPg (Joubert *et al.*, 2000, Konig *et al.*, 1998), and the 3' end is polyadenylated (Boga *et al.*, 1999). The 3' end is predicted to have a 2 stem-loop structure with a free energy of 7.1 kCal/mol. This resembles structures found in the wild type poliovirus and coxsackievirus B1 (Seal *et al.*, 1994). It is likely that this structure interacts with the viral polymerase during RNA amplification.

1.2.4 Phenotypic variations of RHDV

The sgRNA encodes the capsid protein (VP60) and the product of ORF2 (VP10). It appears to produce most of the protein that forms the capsid shell in RHDV virions (Boga *et al.*, 1999). The capsid protein comprises 90 dimers (Martinez-Torrecuadrada *et al.*, 1998) forming a shell domain with arch-like capsomers. The N-terminal region forms the shell and the C-terminal region forms the arches (Granzow *et al.*, 1996, Laurent *et al.*, 2002, Laurent *et al.*, 1997). The N-terminal region is therefore buried beneath the arches and is less accessible to the antibodies of the host animal. During the course of infection it has been shown that two different types of RHDV are found, a wild type RHDV with the ordinary shell and arches and a smooth type RHDV, without the arches formed by the C-terminus of the capsid protein. This is known as sRHDV (Barbieri *et al.*, 1997), or core-like particles (CLP's) (Granzow *et al.*, 1996). The capsid protein of these virions is formed from the N-terminus of the VP60 protein, and is a 30 kDa protein (Granzow *et al.*, 1996). The proportion of sRHDV in samples increases with the length of time of infection. For example, at 70-80 hours post infection (h.p.i.) 40-50% of the RHDV present within the rabbit are sRHDV, and at 140-160 h.p.i. the proportion is 70-80%. Thus, the incidence of this morphologically variant form of RHDV is linked to the occurrence of a chronic or subacute disease status. Currently, there are two possible theories as to the origin of sRHDV, firstly they arise during clearance of the virus by the host animal (Capucci *et al.*, 1991), or secondly, they represent virions with truncated VP60 protein (Barbieri *et al.*, 1997). In experimental infections it has been shown that sRHDV (or CLP's) behave as normal caliciviruses (Schirrmeier *et al.*, 1997). It is therefore considered more likely that the sRHDVs result from truncated genome expression rather than proteolytic clearance by the

host. It is possible that these sRHDVs correspond to a less pathogenic form of RHDV and infection with sRHDV may lead to a milder infection.

The RHDV VP60 region appears to be the most antigenically variable protein found in RHDV (Martinez-Torrecuadrada *et al.*, 1998, Viaplana *et al.*, 1997). The N-terminal part of the capsid protein, i.e. the inner shell of the virion appears to be more antigenic than the protruding arches of the C-terminal region of the VP60 protein. The capsid protein appears to be highly conserved (Schirrmeier *et al.*, 1999), yet within this region there is sufficient variation for discrete subpopulations and quasispecies to be apparent (Gould *et al.*, 1997). Several distinct antigenic variants have been identified: in Italy two have been isolated, RHDVa (Capucci *et al.*, 1998), and Rabbit Calicivirus (RCV) (Capucci *et al.*, 1996b). RHDVa varies by 6.9% from the more common RHDV seen in Italy, whilst RCV is a non-pathogenic virus that varies by 14.6%, but still induces protective antibodies in rabbits exposed to virulent RHDV. RCV is primarily found in the large intestine of the rabbits rather than the liver. Under experimental conditions German antigenic variants cause lethal disease in spite of the variation in morphological and biological properties of the strains (Schirrmeier *et al.*, 1997). Therefore RCV appears to be the only distinct variant of RHDV identified to date.

It is thought that the different surface appearance of sRHDV means it is more likely to elucidate B lymphocyte humoral response. How the virus persists during sub-acute or chronic infections is yet to be decided. One possibility is that the protruding arches of the capsid protein protect the virus from antibody attack, thus mediating rapid infection and death. Thus, rabbits that survive infection by RHDV may have more of the sRHDV form therefore induce a more effective immune response leading to sub-acute or chronic infection.

1.2.5 Pathology of RHDV

Classically, RHDV causes acute infection in rabbits, killing them 1-3 days post infection (p.i.). The animals exhibit acute fulminating hepatitis, involving the liver, also involving lungs and spleen (Park *et al.*, 1995). Shortly before death they often show signs of anorexia and lethargy, and animals die in a characteristic position (Fig 1.2).

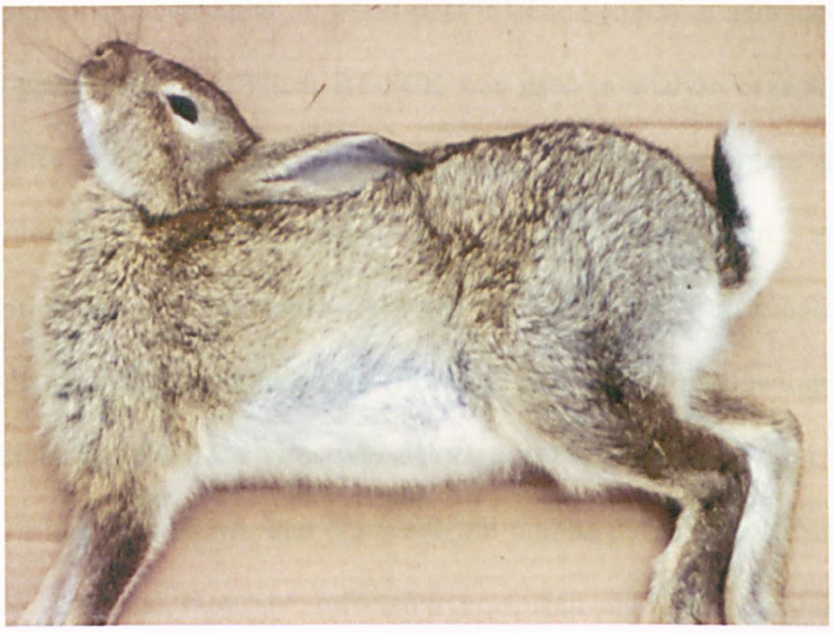

Figure 1.2
Showing the characteristic position assumed by rabbits that died of RHDV (picture courtesy of Dr. R. Trout)

The coexistence of positive and negative-stranded viral RNA in the liver, lungs and spleen indicates that viral replication occurs in these tissues (Kimura *et al.*, 2001). During the course of the infection the liver becomes necrotic with infiltration of heterophils (Jung *et al.*, 2000). As well as acute fulminating hepatitis, there is obstruction of the blood vessels (Alonso *et al.*, 1998, Tunon *et al.*, 2003) in the

suprahepatic, aortic and pulmonary vessels, with the most severe lesions being found in the liver and the lungs (Ramiro Ibanez *et al.*, 1999). Macrophages in these tissues are infected by 12 h.p.i., and this macrophage and liver tropism characterises RHDV. During the terminal stages of the disease disseminated intravascular coagulation (DIC) occurs. It is thought that the initial hepatic injury causes the DIC and subsequent haemorrhages in other organs (Ramiro Ibanez *et al.*, 1999, Ueda *et al.*, 1992). In laboratory rabbits infected orally death occurred 1-3 days post infection (Guittre *et al.*, 1996). The liver was the primary histopathological site, with the most severe hepatitis occurring at 70 hours post infection (h.p.i) in animals that survived for this period of time. When RT-PCR was used to analyse organs the liver and spleen were found to be infected by 18 h.p.i., with all major organs infected by 36 h.p.i., lesions appeared 12-18 hrs later. Subsequent studies using immunofluorescence microscopy showed that the liver can support replication almost immediately after infection (Prieto *et al.*, 2000). Following subcutaneous inoculation of rabbits, the virus was detected in the liver by 18 h.p.i. (Shien *et al.*, 2000). It has been shown that 4-5 week-old rabbits do not usually succumb to the virus. Nevertheless, they have infected liver, spleen and bile as early as one day p.i., and all organs contain the virus 2 days p.i. However, no virus was detected in the rabbits 7 days p.i. These observations led to the proposal that young rabbits have an efficient clearing mechanism not seen in mature rabbits (Shien *et al.*, 2000).

1.2.6 The Origin of RHDV

The first recorded epidemic of RHDV was observed in China in 1984, where the initial epidemic killed an estimated 470,000 domestic rabbits in 6 months (Carman *et al.*, 1998), and the virus dispersed approximately 50,000 km^2 from the

initial focus within 9 months (Gregg *et al.*, 1991, Xu, 1991). With hindsight it is possible that the disease was introduced into China by the importation of Angora rabbits from Germany. However, since the German Angora rabbits were apparently healthy at the time of their introduction into China, it could be argued that they carried the virus in a "silent" form. Of course this could equally apply to the rabbits already present in China. Therefore there are at least two possible explanations as to how the virus suddenly became highly virulent. Firstly, the Chinese population of domestic rabbits could have been immunologically totally naïve to RHDV. This might render them highly susceptible to an introduced virus that was not causing overt disease in German rabbits that were presumably not immunologically naïve to the virus. A second possibility is that recombination occurred between the German strain of virus and an indigenous Chinese strain causing a significant shift in the virulence of the recombinant virus leading to the severe epidemic seen in 1984. Whilst recombination has never been proposed previously, evidence will be presented in this thesis to demonstrate that recombination does occur between strains of RHDV. From China it was assumed that the newly virulent virus dispersed across Asia and Europe because a similar virus was isolated from fatally infected rabbits in Italy in 1986 (Cancellotti & Renzi, 1991) but it was labelled 'Malattia-X'. The virus was also reported in the former Czechoslovakia in 1987 (Smid *et al.*, 1989), and Poland and Germany in 1988 (Gorski *et al.*, 1994, Loliger & Eskens, 1991). The virus in Czechoslovakia was found to be the same as that isolated in China in 1984. In Poland the rapidity of the spread of RHDV equalled that seen in China. There was significant but varying mortality among the domestic rabbits farms ranging from a few fatalities to 99.5% mortality rates (Gorski *et al.*, 1994). By 1996 epidemics due to RHDV were present in over forty countries (Heneidi Zeckua *et al.*, 1997),

mainly in domestic rabbits including countries from the Middle East (Abu Elzein & al-Afaleq, 1999), Africa (Bouslama *et al.*, 1996, Kpodekon & Alogninouwa, 1998), Mexico (Gregg *et al.*, 1991, Heneidi Zeckua *et al.*, 1997) and the Far East (Berninger & House, 1995, Mizoguchi *et al.*, 2003).

The first case of RHDV in wild rabbits was reported in Spain in 1988 (Cooke, 2002, Villafuerte *et al.*, 1994). The virus subsequently emerged in wild rabbit populations in France (Marchandeau *et al.*, 1998, Marchandeau *et al.*, 2000), the UK (Fuller *et al.*, 1993, Patterson & Howie, 1995) and Ireland (Collery *et al.*, 1995, Graham *et al.*, 1996, Sammin *et al.*, 1995). Since this time the virus has become established among many of the wild rabbit populations of Europe.

Amongst domestic rabbit populations the virus can be controlled by vaccination (Bouslama *et al.*, 1996, Gorski *et al.*, 1994) and can even be eradicated (Heneidi Zeckua *et al.*, 1997). However, amongst wild rabbit populations it is impossible to control the virus and it has continued to circulate throughout Europe with epidemics and many deaths constantly being recorded.

Since the emergence of RHDV in 1984 several retrospective studies have shown the presence of antibodies in sera collected before 1984 (Moss *et al.*, 2002, Nagesha *et al.*, 2000, Nowotny *et al.*, 1997, O'Keefe *et al.*, 1999, Robinson *et al.*, 2002a, Rodak *et al.*, 1991). Thus prior to 1984, RHDV appears to have been circulating amongst rabbits without causing disease. Whether or not RHDV circulated in an avirulent form has never been absolutely demonstrated since no infectious virus has been isolated from pre-1984 archival material. An equivalent situation appears to have pertained in New Zealand and Australia, prior to the deliberate release of RHDV in these countries, i.e. archival rabbit sera were positive for RHDV (Nagesha *et al.*, 2000, O'Keefe *et al.*, 1999, Robinson *et al.*, 2002a). A

plausible explanation for this followed the isolation of Rabbit calicivirus (RCV) in domestic rabbits in Italy in 1996 (Capucci *et al.*, 1996b). Rabbit calicivirus is antigenically cross-reactive with RHDV and following infection, protects rabbits from challenge with virulent strains of RHDV. Moreover sequence data confirmed the genetic relationship between RCV and RHDV (Capucci *et al.*, 1996b) although they are clearly distinct in evolutionary terms when analysed phylogenetically (Moss *et al.*, 2002). A particularly interesting observation was made when stored rabbit sera dating back to 1955 were analysed for the presence of RHDV antibody (Moss *et al.*, 2002). These rabbits sera contained RHDV-specific antibodies in ELISA tests but the sequences of the RNA detected in these sera were variable but very similar to a wide range of RHDV strains from the UK and Europe. Moreover, they resembled the sequences of strains isolated as recently as 2000, the most recent strains analysed. It is important to note that they were much less closely related to RCV. Thus, it appears that the viruses circulating prior to 1984 were not simply avirulent viruses that were superseded by virulent viruses in 1984. The evidence of RHDV circulating in an apparently silent form in rabbits prior to 1984 raises questions about the mechanism by which the disease appeared so dramatically and the virus subsequently appeared to disperse across Asia and Europe. There are several mechanisms by which a virus may suddenly emerge as a new disease agent. Firstly, under strong selection pressure, such as that seen when SIV mutated to become HIV (Chen *et al.*, 1997, Sharp *et al.*, 1999, Sharp *et al.*, 1995). Secondly, many gradual neutral mutations may accumulate to cause a significant change in the phenotype of the virus, this is known as antigenic drift (Yin *et al.*, 2004). Antigenic drift is unlikely to be the cause of epidemic RHDV emergence because Moss *et al* (2002) showed that the sequence similarity between viruses from 1955 and 2000 is very

high. Thirdly recombination may take place. Although recombination has not yet been formally demonstrated in RHDV, the prevalence of recombination in other positive stranded RNA viruses suggests that this is a likely scenario for the significant shift in the virulence of the virus in 1984 (Dahourou *et al.*, 2002, Santti *et al.*, 1999, Twiddy & Holmes, 2003, Uzcategui *et al.*, 2001, Weaver *et al.*, 1997, Worobey & Holmes, 1999) and as will be shown in this thesis, it does occur. Finally, the virus could have been causing periodic outbreaks of disease of varying severity for a significant period of time, and the outbreak in China was an extreme recurrence of this epidemicity.

1.2.7 The impact of RHDV in Europe

An intriguing characteristic of RHDV is the varying patterns of epidemiology in different ecological regions throughout the world. During the initial epidemic in China in 1984 the mortality rate was about 80% of the domestic rabbit population. This high mortality rate has been relatively consistent amongst domestic rabbits globally. However, the wild rabbit populations of Europe show a wide range of epidemiological patterns. In Spain 55% of the wild rabbit population was killed during the first recognised epidemics, and similar numbers were reported in France (45%) (Marchandeau *et al.*, 1998). These mortality rates have continued leading to a significant decline in the number of rabbits in Spain with major implications for predators of rabbits such as the Iberian Lynx (*Lynx pardinus*) and the Imperial Eagle (*Aquila adalberti*) (Angulo & Cooke, 2002).

In common with many other countries, following the first appearance of disease, the UK initially witnessed high mortality rates (80%) amongst domestic rabbits (Fuller *et al.*, 1993), and the virus subsequently spread throughout the wild

UK rabbit population. Although there were major epidemics, with high mortality rates, the spread and distribution of these outbreaks did not conform to a uniform pattern. The virus appeared to cause outbreaks randomly throughout the countryside with geographic separation as high as 150km between outbreaks. Moreover, in some outbreaks the mortality rate was high but in neighbouring populations there was apparently no disease at all (R. Trout – personal communication). One possible explanation for this arose from a study in which it was shown that up to 60% of the wild rabbit population in the UK had antibodies to RHDV (Trout *et al.*, 1997b), possibly preventing the devastating epidemics seen in Australia where immunity was either totally absent or present only at a low level.

1.2.8 RHDV as a Bio-control Agent

The observed and recorded high virulence of RHDV in Asia and Europe led to it being considered as a bio-control agent in Australia, where the European rabbit, a recent coloniser of Australia following its introduction in the 1850's had become a major pest destroying the local flora and fauna (Asgari *et al.*, 1999, Sandell, 2002). The introduction of Myxoma virus (MYXV) in the 1950's had initially reduced rabbit populations, but the virus gradually became attenuated and the rabbit population recovered to pre-myxomatosis levels (Saint *et al.*, 2001). In order to test the efficacy of RHDV for controlling rabbit populations field trials were organised on Wardang Island, 5km off the southern coast of Australia. Among other things, these trials demonstrated that the European rabbit was the only susceptible host for RHDV (Lenghaus *et al.*, 1994). During the course of the trial the virus escaped from Wardang Island onto mainland Australia. Due to the unusual nature of this virus release onto the Australian mainland, the spread of the disease was monitored

closely. The virus escaped from Wardang Island in September 1995. The virus spread amazingly quickly through the rabbit population, rates varied from 9 km/month during the summer and to 414 km/month during the spring (Kovaliski, 1998). By the summer of 1995/1996 the percentage of surviving rabbits with RHDV-specific antibodies was 80%, and 20 million rabbits had been killed in the North-eastern part of Southern Australia. However, on the periphery of the epidemic, the percentage of rabbits with antibodies to the virus, was only 20% (Cooke, 1997). In areas of high rabbit density the initial epidemics reduced the rabbit population by 95% (Mutze *et al.*, 1998). By the end of October 1996 the virus was present in 50% of mainland Australia (Kovaliski, 1998). The spread of the virus was phenomenal with average distances of 10-18km per day, compared to 15 km per month in Spain (Cooke, 1997). This rapid spread was in no small way aided and abetted by farmers who deliberately released the virus in order to control the rabbit population.

In subsequent years following its release into Australia it has become clear that the impact of the virus on rabbit populations is largely dependent on environmental factors. In semi-arid regions the virus appears to have had a major impact on rabbit populations, with high mortality rates and a relatively low incidence of seropositive rabbits (Cooke *et al.*, 2002). In wetter areas such as the eastern and western coastal regions of mainland Australia, RHDV mortality rates have been much lower (Henzell *et al.*, 2002), and a larger proportion of the rabbits are seropositive to RHDV (Cooke *et al.*, 2002). These latter areas usually have an annual rainfall of 400-500mm. Another consequence of the introduction of RHDV to Australia is the altered rabbit breeding patterns and therefore the occurrence of myxomatosis in Australia. The magnitude of this impact is also dependent on the

climate and rabbit population density, but RHD generally depresses the rabbit populations during the breeding season and this delays the outbreaks due to myxomatosis (Mutze *et al.*, 2002). This is most likely due to the difference in virus incubation times, with RHDV outcompeting the MYXV within the rabbit populations. Henzell *et al* (2002) concluded that the impact of RHDV depended on the following factors:

1. RHD outbreaks are less effective where there are low densities of susceptible rabbits
2. Once an outbreak of RHD has occurred, the disease will cause high mortality in winter rainfall areas, and hot dry areas throughout the year, although the probability of an outbreak in summer is lower
3. RHDV outbreaks are relatively less effective in cold/wet areas

Although New Zealand had a similar problem with rabbits impacting significantly on the flora and fauna, the New Zealand government was initially reluctant to introduce RHDV by deliberate release. A "wait and see" policy was adopted, whilst the situation in Australia was closely monitored. However, in 1997 the New Zealand farmers took matters into their own hands and deliberately released the virus onto the farmland. Infected carcasses obtained from Australia were macerated and used to lace bait such as oats and carrots. The virus rapidly became established in the rabbit populations and the government eventually sanctioned a controlled release in order to avoid the illegal releases from running out of control (Motha & Clark, 1998).

Recent studies have shown that the deliberate release of RHDV in New Zealand has had at least three different types of impact. Firstly, in some areas, a

high proportion of rabbits have RHDV-specific antibodies as the result of being exposed to, but surviving infection by the released virus. In these areas epidemics due to RHDV are not recorded, and the rabbit populations have stabilised. This has been observed in northern Canterbury, a climate similar to the coastal areas of Australia. Secondly, in other areas, epidemic outbreaks have reduced the number of rabbits and they have then failed to recover to their previous levels. This has been typically observed in Central Otago, and the Mackenzie basin, both semi-arid climates. Thirdly, epidemics occur, reducing the rabbit population but they gradually recover to reach pre-RHD levels (Parkes *et al.*, 2002). In common with many areas in Australia, the virus in Central Otago appears to have become established as an endemic virus, with annual epidemic outbreaks (Barlow *et al.*, 2002).

In both Australia and New Zealand the initial effect of deliberately releasing RHDV as a bio-control agent was quite promising in that a significant proportion of the rabbit population died. However, this trend has not been maintained and in many places the rabbit populations are recovering to their pre-RHDV levels, although this is not the case everywhere: in some areas the released virus has circulated continuously thus keeping down the rabbit population. By analogy with the situation in Europe, it is likely that the presence of pre-existing viruses in both New Zealand and Australia (Nagesha *et al.*, 2000, O'Keefe *et al.*, 1999, Robinson *et al.*, 2002a) have had a significant impact on the epidemiology of the virus.

This raises interesting questions. Firstly how does the virus spread, and is the presence of different vectors responsible for the variety of epidemiological patterns reported? Secondly, does the sequence variation seen in RHDV affect the epidemiology? Finally, if as retrospective studies suggest, pre-existing viruses were

present and not causing epidemics how does this influence the spread and virulence of RHDV in wild rabbit populations? The work described in this thesis addresses the last two questions in particular.

1.2.9 Diagnosis of RHDV

Although RHDV is considered to be a lethal disease of rabbits, killing a high proportion of individuals within 2-3 days of infection, a proportion of rabbits survive to produce protective antibodies. Initially, the presence of RHDV in rabbit liver was detected by the detection of Haemagglutinin (HA), using group O erythrocytes, and the RHDV-specific antibody status was analysed using the haemagglutination-inhibition (HAI) test. This test has proven to be robust. Nevertheless, several strains have been found that do not haemagglutinate erythrocytes at 20°C. For example the strain known as Rainham haemagglutinates human erythrocytes at 4°C (Capucci *et al.*, 1996a), whereas some strains do not haemagglutinate human erythrocytes at all (Schirrmeier *et al.*, 1999). In many laboratories the HA test has been superseded by the Enzyme Linked Immunosorbent Assay (ELISA) (Collins *et al.*, 1995), which identifies capsid and other virus-specific antibodies in the serum of rabbits. It is a matter of debate whether or not ELISA provides more useful scientific information than the HAI test but because it can be produced in a stable kit form, it is undoubtedly more amenable to the analysis of large numbers of samples by inexperienced scientists. Certainly with the presence of strains of RHDV that do not haemagglutinate, or haemagglutinate at different temperatures, the ELISA is less likely to give false negative results. The ELISA test also allows, in limited form, the identification of different serotypes of the virus (Capucci *et al.*, 1991). In contrast with the detection of antibodies against RHDV, the reverse-transcriptase polymerase

chain reaction (RT-PCR) detects viral RNA. This test is highly sensitive and has completely altered our ability to (a) detect the presence of the viral RNA in rabbit tissues/organs/sera and (b) to compare the sequence of the RNA of individual strains of RHDV that we have begun to realise exist in rabbits populations in different areas of Europe and Asia. Indeed, used skilfully, the RT-PCR can provide an indication of the relative quantity of viral RNA present in the liver. Unfortunately, RHDV cannot be cultured in cell monolayers, it is therefore not possible to carry out standard infectivity assays and this could be one of the several possible reasons the presence of RHDV-specific RNA fragments and even complete genomes in healthy rabbits have only recently been identified (Forrester *et al.*, 2003, Moss *et al.*, 2002).

1.2.10 Epidemiology of RHDV and the antibody response to infection

Initial studies in wild rabbit populations have shown that RHDV is linked to the breeding season in rabbits. This breeding season is itself dependent on the growth of vegetation, which is linked to the annual rainfall and temperature of the environment in which the rabbits find themselves (Wood, 1980). Epidemics of RHDV have been shown to occur as a result of the recruitment of new susceptibles into the population as a result of the breeding season (Calvete *et al.*, 2002, Cooke, 2002, Simon *et al.*, 1995, Villafuerte *et al.*, 1994). If, for some reason, such as low rainfall, the breeding season is delayed, the corresponding RHD epidemic will also be delayed (Calvete *et al.*, 2002). In studies in Spain, the epizootics were shown to last for 4-5 weeks; with the epidemics affecting some but not all social groups of rabbits. If the outbreaks occurred early in the breeding season, the disease affected the young sero-negative rabbits that had survived from the previous years' breeding season, and this resulted in the deaths of some of the younger rabbits. Outbreaks

later in the season primarily affected the juveniles that no longer had the benefit of maternal antibody protection (Calvete *et al.*, 1995, Cooke, 2002).

Rabbit populations are subject to high levels of mortality, in the main from predation and disease. In Spain it was found that the presence of RHDV among the wild populations meant that deaths from disease in adults were attributed to RHD rather than any other disease, whereas juveniles were considered more likely to succumb to myxomatosis rather than RHDV. All adults tested had antibodies MYXV. The advent of RHDV has increased the mortality rate, such that in areas of low density, rabbit populations are declining. It has been argued that the mortality rate of RHDV should increase with population density, partly because of the increased contact between members of the population in an high density rabbit population (Cooke, 2002). However, evidence from the field has suggested otherwise. Outbreaks in areas of high rabbit density lead to reduction in median age of the animals that are exposed to the virus. Thus, rabbits are exposed during the phase when they are protected by maternal antibodies and more rabbits survive to the next breeding season. Therefore the impact of the disease is mitigated. In populations where there is lower rabbit density, the disease can cause such a reduction in the recruitment of juveniles into the breeding population that the population declines severely (Cooke, 2002).

The fact that RHDV occurs in an annual cyclical pattern means that it must be an endemic disease (Marchandeau *et al.*, 1998). Thus, somehow the virus presumably persists in the environment between the epidemic outbreaks. There are reports of deaths from RHDV occurring throughout the year, in the absence of obvious epizootics (Marchandeau *et al.*, 1998, Simon *et al.*, 1995). However, most fatal infections occur during the epizootics.

Survivors of the outbreaks are found to have antibodies to RHDV, and it has since been determined that in most populations a proportion of rabbits do not become sick and die as the result of infection by RHDV. Initial experiments using HAI tests showed a relatively high prevalence of RHDV-specific antibodies in rabbit populations. In fact studies have shown that the proportion of rabbits with antibodies can vary from 22.9% to 70% (Cooke *et al.*, 2000, Schirrmeier *et al.*, 1997, Trout *et al.*, 1997b). These results were taken from populations that were not known to have been exposed to RHDV. After an epidemic this level of antibody prevalence can then increase to 100% (White *et al.*, 2004). In every population studied there has been a significant prevalence of RHDV-specific immunity in the population in the absence of overt disease (Moss *et al.*, 2002, Nagesha *et al.*, 2000, O'Keefe *et al.*, 1999, Robinson *et al.*, 2002a, Rodak *et al.*, 1990). It has been demonstrated that it is possible for rabbits to seroconvert in the absence of overt disease (Capucci *et al.*, 1997). However, recent work suggests that the presence of antibodies does not always result in protection against a fatal infection caused by RHD (Calvete *et al.*, 2002, Marchandeau *et al.*, 2005).

The use of ELISA tests has meant that different types of antibodies can be detected. The use of anti-IgG, IgM and IgA enables the stage of infection of the rabbit to be determined. IgM antibodies are present during the initial stages of infection of RHDV within the rabbit, an IgA response indicates that the rabbit has been re-exposed to the virus, and an IgG response indicates a long-term antibody presence (Cooke *et al.*, 2000). In juvenile rabbits between 2-8 weeks of age, the presence of IgG is almost certainly the result of maternal antibodies derived from milk. In rabbits less than approximately 9 weeks of age, there is a reduced mortality rate compared with adults exposed to RHDV. This is assumed to be partly due to the

presence of RHDV-specific antibodies in the juveniles, but also partly due to a form of natural resistance that has not yet been adequately defined. The length of time over which the antibodies persist in the juveniles is dependent on the titre of antibodies in the mother (Robinson *et al.*, 2002b). The rabbits are demonstrably immunologically competent at around 4-6 weeks *postpartum*, by which time the contributed maternal antibodies have significantly reduced leaving the juveniles susceptible to RHDV (Cooke *et al.*, 2000, Mikami *et al.*, 1999). Although declining maternal antibodies may partly explain the developing susceptibility of the rabbits as they grow older, another possible factor relates to the physiological changes that occur in the liver during the weaning process, i.e. as the rabbits switch from milk to a solid diet (Balistreri & Schubert, 1987). It is possible that this change in liver physiology renders the non-immune animals susceptible to RHDV.

In order to understand the process by which animals become immune to RHDV, and the effect this has on the population, a long-term capture-recapture study of wild rabbits and the resulting antibody response needs to take place. This could provide a long-term view of the ecology, the immunology and the population dynamics of the rabbits in the presence of RHDV.

1.2.11 The Spread of RHDV

At the present time the mechanism of transmission of RHDV is unknown. There are several possible alternatives: RHDV has been shown to be transmitted orally between rabbits (Capucci *et al.*, 1997, Heneidi Zeckua *et al.*, 1997). However, this would not account for the ability of the virus to jump great distances, as seen in the UK, or indeed the rapid spread of the virus recorded in Australia when it escaped from Wardang Island. RHDV has been shown to be present in rabbit faeces and

decomposing rabbit carcasses (McColl *et al.*, 2002b, Shien *et al.*, 2000), and this could contribute to the longevity of the epidemics. Myxomatosis virus may also be transmitted by mosquitoes that carry the virus mechanically on their mouthparts, because of this there have been several efforts to determine whether or not there is a significant insect vector for RHDV. Recent work demonstrated that in Australia, Bushflies (*Musca vetustissuma*) increased 1000-fold during the period when the virus escaped onto the Australian mainland, and when the Bushflies were tested, RHDV was found in 4/10 of the samples (McColl *et al.*, 2002a). Rabbit fleas, *Spilopsyllus cuniculi* and *Xenopsylla cunicularis* and the mosquito *Culex annulurostris* have also been shown to be carriers and transmitters of RHDV (Lenghaus *et al.*, 1994). Nevertheless in Europe, there have been virtually no studies on insect transmission of RHDV. However, the difference in rate of spread of RHDV in Australia, 10-18km/day, compared with 10-15km/month in Spain, suggests that if insect vectors are important agents of dispersal there are, not surprisingly, significant differences between the vectors in Australia and Europe. In the UK it is believed that seabirds and carrion birds may be able to carry the virus relatively long distances (Chasey, 1994). This is not inconsistent with recent work showing that the virus can survive up to three weeks in decomposing rabbit carcasses (McColl *et al.*, 2002b) and in rabbit bone for even longer (Moss *et al.*, 2002). There is also earlier evidence of genetically closely related strains of RHDV in geographically separated rabbit populations throughout mainland Britain and the continent of Europe (Le Gall *et al.*, 1998, Moss *et al.*, 2002).

1.2.12 Molecular Epidemiology of RHDV

RT-PCR sequencing has greatly facilitated the determination of RHDV sequences. Phylogenetic analysis of these viruses using comparative alignments has enabled us to identify the possible origins and relationships between different RHDV isolates obtained from around the world. Detailed analysis of these viruses immediately revealed that they could disperse at enormous speeds and over significant distances. For example, in France, samples from 1988-1995 showed clustering by year rather than by geography, supporting the idea that the virus was easily disseminated over large distances (Le Gall *et al.*, 1998). The three groups of RHDV strains identified in France correspond to a possible introduction from Spain, Germany or Czechoslovakia, and the UK or Italy. In the UK, Moss *et al.* (2002) identified three genogroups circulating within the UK; one group comprised only UK samples collected from either healthy or dead/infected rabbits, over a period of more than 40 years between 1955 and 2000. The viral RNA sequence obtained from healthy rabbits was virtually indistinguishable from that obtained from dead rabbits. Moreover in the other groups, other UK viruses from the 1970's and 1950's showed very close genetic relationships with viruses obtained from various countries in Europe clearly implying that RHDV had circulated harmlessly throughout Europe (and Asia) prior to the first recognised epidemic in China (Liu *et al.*, 1984).

Phylogenetic analysis also revealed that two strains of RHDV, Rabbit Calicivirus (RCV) (Capucci *et al.*, 1996b) an Italian isolate, and Ashington (Moss *et al.*, 2002) a British strain, are genetically very different from all of the other RHDV isolated thus far (their ancestral lineage is much more divergent than the other viruses) and also widely different from each other. Interestingly RCV is a non-pathogenic strain and Ashington was isolated from a rabbit that died of clinically

typical haemorrhagic disease implying that it is a pathogenic strain. One obvious question that immediately springs to mind is whether or not these two very different strains of RHDV contribute significantly to the epidemiology of RHD.

In Australia, where a single strain of virus escaped onto the mainland from Wardang Island, sequence analysis was used to monitor the rate of genetic variation following the escape (Asgari *et al.*, 1999). Periodically from 1995 to 1997 virus isolates were collected from rabbits in the epidemic pathway and their sequences were determined. Over two years of study a maximum variation of 1.8% was identified. There was no clear evidence of geographic clustering. These results suggest that the rate of change in the virus is significantly constrained. If this observation is truly representative, then the relatively high level of divergence seen amongst different strains of RHDV isolated in Europe and Asia infers either that RHDV must have been circulating within rabbit populations for many years or the rates of variation vary under different epidemiological circumstances. These observations also support the proposal that RHDV emerged many years prior to the 1984 epidemic in China. How the emergence of the Chinese strain in 1984 fits into this picture, and the means by which the virus was able to maintain itself within the population with no overt symptoms of disease are some of the questions tackled by this thesis.

1.2.13 Possible Mechanisms of Persistence for RHDV

It has previously been stated that the phylogenetic evidence leads to the inference that the virus has been circulating within the rabbit populations for at least 50 years. Moreover, the length of time between outbreaks seen especially in wetter areas suggests that the virus must persist both in the long- and short-term. There are

two ways by which RHDV may persist. Firstly, it may persist in the environment, and secondly, the virus may persist within the rabbit. So far, no other carriers of the virus have been identified despite extensive field studies in Australia. Therefore, it appears that a secondary host cycle can be ruled out (Lenghaus *et al.*, 1994).

RHDV persists for a period of time in decomposing rabbit carcasses and the faeces of infected rabbits (McColl *et al.*, 2002b, Shien *et al.*, 2000). If the rabbits die above ground then the virus is able to persist for at least 6 weeks. However many rabbits die below ground (Cooke, 1997) and thus rabbit burrows remain a good source of the virus (Trout *et al.*, 1997a). Most likely, RHDV is found in both the decomposing carcasses of infected rabbits and the faeces within the burrows. It is also possible that the presence of diseased animals within the burrows provides an added source of infection either by aerosol droplets or through contact. Decomposing carcasses both above and below ground allows predators and insects access to the virus and they may also be carriers of the virus. Predators e.g. foxes (*Vulpes vulpes*), have been shown to carry the virus (Frolich *et al.*, 1998, Leighton, 1995), but whether this gives rise to further infections in rabbits is unknown. Insects that inhabit rabbit populations are thought to be highly effective in aiding virus dissemination. For example, the presence of large numbers of the bush fly (*M. vetustissuma*) permits rapid and widespread RHDV infection in Australia (Kovaliski, 1998). Although nine species of fly have been demonstrated to carry the virus (Asgari *et al.*, 1998), the bush fly and the rabbit flea (*S. cuniculi*) are thought to have the most impact in disease transmission. However, it is now known that insects cause infection via mechanical transmission rather than acting as a viral reservoir (Asgari *et al.*, 1998, McColl *et al.*, 2002a). In spite of this insects are integral to the

spread of RHDV especially in Australia and New Zealand (Heath *et al.*, 1999, Kovaliski, 1998, Lugton, 1999).

The virus may persist within the rabbit population by causing continuous low level disease. In other words, a small but significant number of rabbits may be infected throughout the year, but epidemics are seen only when there is an increase of young rabbits during the breeding season. There is some evidence of all year round low level mortality due to RHDV (Cooke, 1997, Marcato *et al.*, 1991). However, the effects of predation are often likely to mask this effect and as yet there is no definitive evidence to support this.

Finally, and possibly the most likely scenario, the disease may survive long-term in the rabbit as a persistent or latent infection. It has previously been suggested that the presence of virus in the environment must be due to the shedding of the virus by a carrier (Calvete *et al.*, 2002, Cooke, 2002). Recent evidence showing the presence of viral RNA in healthy rabbit livers and stored rabbit sera (Forrester *et al.*, 2003, Moss *et al.*, 2002, Zheng *et al.*, 2002) has confirmed that RHDV RNA can survive long-term in the liver. Whilst this has not yet been shown to be infectious, the detection of genomic length RNA in healthy rabbits offers a plausible explanation for the persistence of RHDV in the absence of epidemic outbreaks. Thus far, no mechanism has been demonstrated to show how virus could survive long-term in the liver. However, in addition to the suggestions above, there are other possibilities based on what we know about other viruses.

Firstly, the virus may circulate as an enzootic form causing no disease and then, following appropriate mutations, emerge as an epizootic form. This is believed to occur with Venezuelan equine encephalitis virus (VEEV) (Johnson & Martin, 1974, Kinney *et al.*, 1992a, Kinney *et al.*, 1992b, Rico-Hesse *et al.*, 1988, Walton &

Grayson, 1988). A single nucleotide substitution in the VEEV envelope glycoprotein causes an increase in virulence upon infection in either horses and humans (Brault *et al.*, 2002). Usually these epidemics are short-lived. The non-mutated enzootic strain continues to circulate in the adjacent forest environment. A second example is provided by the discovery of a non-pathogenic rabbit calicivirus (RCV) (Capucci *et al.*, 1996b). This virus, which has a significantly different genomic sequence from recognised virulent strains, circulates harmlessly amongst domestic rabbits in Italy, presumably immunising the rabbits that it infects (Capucci *et al.*, 1996b). More recent work on RHDV isolates from New Zealand and from the UK has shown the presence of viral RNA in healthy rabbits. The sequence of this viral RNA is very similar (97.7%) to that of strains isolated from rabbits that died following infection with RHDV (Forrester *et al.*, 2003, Moss *et al.*, 2002, Zheng *et al.*, 2002). More genomic-length sequences are required to establish whether or not common mutations are present in strains that do not appear to cause overt disease.

If RHDV does persist in a silent form long-term in healthy rabbits, as inferred by Zheng *et al* (2002) and Forrester *et al* (2003), we need to know how it achieves this. How, for example, is the viral RNA able to withstand degradation by nucleases? Viruses in the genus *Flavivirus* produce replicative forms (RF) of RNA, which with the replicative intermediate (RI) and the nascent single-stranded RNA tails become part of the replicative complex (RC) (Uchil & Satchidanandam, 2003a, Uchil & Satchidanandam, 2003b, Westaway, 1987). In order to facilitate replication, these viruses rearrange host-derived membranes, to produce distinct vesicle-like packets (VP) which house the RF (Westaway *et al.*, 1999, Westaway *et al.*, 1997). The RFs are bound by proteins that protect them from nuclease degradation. In this form they are highly stable and may be able to persist within the host for a significant

period of time. Other RNA viruses, such as picornaviruses, togaviruses, coronaviruses, bromoviruses and arterioviruses, manipulate host membranes during replication (Egger *et al.*, 1996, Magliano *et al.*, 1998, Restrepo-Hartwig & Ahlquist, 1996, Schaad *et al.*, 1997, Shlegel *et al.*, 1996, Strauss & Strauss, 1994, van der Meer *et al.*, 1998, Wimmer *et al.*, 1993). Although this has not yet been demonstrated for caliciviruses, the RNA-dependent RNA-polymerase (RdRp) of RHDV is structurally similar to that of the flavivirus Hepatitis C virus (HCV) despite low sequence homology (Love *et al.*, 2004). Thus, the RNA of RHDV may be protected in rabbit liver cells by a similar mechanism to that employed by many other RNA viruses.

The final possibility is that the presence of endogenous reverse transcriptase within the host can produce a dsDNA molecule from the ssRNA viral molecule, and it is this DNA form that may allow integration into the host genome (Zhdanov, 1975). The presence of this form of DNA has been demonstrated in the members of the *Flaviviridae* (Crochu *et al.*, 2004, Drynov *et al.*, 1981), the *Paramyxoviridae* (Zhdanov, 1975, Zhdanov & Pafanovich, 1974), the *Togaviridae* (Zhdanov & Azadova, 1976), and the *Arenaviridae* (Gaidamovich *et al.*, 1978, Klenerman *et al.*, 1997).

Recently, Crochu *et al* (2004) have demonstrated that the genomes of *Aedes aegypti, Aedes albopictus* and *Anopheles gambiae* had several viral regions integrated into the DNA with the sequence being similar to Kamiti River virus (KRV) (Crabtree *et al.*, 2003, Sang *et al.*, 2003) and cell fusing agent virus (CFAV) (Cammisa-Parks *et al.*, 1992). The integrated sequences showed around 60% homology with the KRV and CFAV and was named cell silent agent (CSA). The

difference in flanking sequences showed that it was likely that the virus had integrated more than once, and suggested that these types of events were more frequent than had previously been thought. Although none of these mechanisms have been demonstrated for RHDV it is likely that one of these may be found to be the mechanism by which RHDV survives long-term within the rabbit.

1.3 European Brown Hare Syndrome Virus

The most closely related virus to RCV and RHDV is European Brown Hare Syndrome virus (EBHSV). This calicivirus has been known since the 1980's. It infects European Hares (*Lepus europaeus*) and Varying Hares (*Lepus timidus*) (Cancellotti & Renzi, 1991, Gavier Widen & Morner, 1991, Nowotny *et al.*, 1997), and resembles RHDV structurally, morphologically and in its genetic strategy (Laurent *et al.*, 1997). Antigenically EBHSV cross-reacts with RHDV in HAI tests (Chasey *et al.*, 1992), yet recombinant EBHSV does not elicit a protective response in rabbits (Laurent *et al.*, 1997). So far it is unknown if recombinant RHDV can elicit a protective response in hares. However, they are distinct species and this is supported by the evidence that nucleotide sequence alignments show they only share 52.6 - 60% nucleotide identity (Nowotny *et al.*, 1997).

1.4 Myxomatosis

A major factor that undoubtedly influences the spread and effectiveness of RHDV is the simultaneous presence of myxomatosis in the population of rabbits throughout the entire regions where RHDV is found (excepting New Zealand). Myxomatosis is caused by the Myxoma virus (MYXV), which is a member of the *Poxviridae* family, and of the genus *Leporipoxvirus* (Fenner & Ross, 1994).

Myxoma virus is a double-stranded DNA virus with a genome length of 161.7 kb, the typical poxvirus hairpin loops and an inverted terminal repeat of 11.5 kb (Saint *et al.*, 2001). There are three closely related viruses in the leporipoxviruses all of which infect *Sylvilagus* spp, and occur naturally in America. Myxomatosis is a non-lethal disease of the Brazilian tapeti (*Sylvilagus brasiliensis*) and is also found in *S. bachmani* a native of California. In these natural hosts the virus causes a small fibroma located at the base of the ear with no other signs of generalised disease (Regnery & Miller, 1972).

Myxomatosis was first recognised as a lethal disease of the European rabbit in 1896 (Fenner & Ross, 1994, Sanarelli, 1898). During subsequent experimental studies the ability of the virus to kill domestic rabbits was determined. It proved to be so virulent for domestic rabbits that the virus was proposed as a possible bio-control agent to reduce the rabbit population in the wild since rabbits were presenting a serious threat to agricultural productivity. During this time two standardised strains of the virus were isolated the Standard Laboratory Strain (SLS) or 'Moses' strain (Moses, 1911), and the Lausanne strain (Bouvier, 1954). Both of these strains had been shown to be highly virulent for rabbits killing them within 9-13 days of intradermal injection. The major difference between the two strains was that the Lausanne strain caused protuberant lesions on infected rabbits whilst the SLS strain caused flat lesions.

The virus was released onto Australian farmlands in December 1950 and initially killed more than 99% of rabbits causing many localised extinction events (Ratcliffe *et al.*, 1952). The virus in Australia is transmitted by the mosquito (Myers, 1954), in particular *Culex annulirostris* and *Anopheles annulipes*. Unlike arboviruses, myxoma virus does not replicate in the mosquitoes. Therefore

transmission from rabbit to rabbit is referred to as mechanical transmission. The virus in the infected rabbit contacts the feeding parts of the mosquito being transferred to the mosquito from an infected lesion on the rabbit (Day *et al.*, 1956, Fenner *et al.*, 1956). The efficacy of transfer to an uninfected rabbit depends on the original titre of the virus in the skin lesion on which the mosquito feeds. The mosquitoes are an effective means of transmitting the virus long distances as MYXV can persist on the mouthparts of the mosquitoes for at least 220 days (Andrewes *et al.*, 1956). In addition to its release in Australia, the virus was also released in Britain and France. In Britain it was introduced into Kent in 1953, taking two years to disperse throughout the whole of Britain (Armour & Thompson, 1955). One of the reasons why it is thought that the disease dispersed more rapidly across Australia than in the UK is that in the UK the virus is transmitted mainly by the rabbit flea (*Spillopsyllus cuniculi*) (Lockley, 1954, Muirhead-Thompson, 1956), whereas in Australia it is mainly transmitted by mosquitoes. Whilst MYXV survives for up to 100 days on the mouthparts of fleas (Chapple & Lewis, 1965), the rabbit flea often does not feed any more frequently than once per 100 days (Allan, 1956).

Following the deliberate introduction of MYXV into the wild rabbit populations the virus gradually became attenuated. Field isolates of MYXV were graded in virulence on a scale of I to V, grade I being the SLS and grade V only killing > 50% of rabbits. The combination of MYXV attenuation and selection for genetic resistance amongst rabbits led inevitably to an increase in rabbit numbers, and whilst MYXV has continued to circulate amongst rabbits globally it is no longer sufficiently pathogenic to impact significantly on rabbit population density.

A question as yet unanswered is whether or not MYXV and RHDV interact, and if so how do they impact on each other?

1.5 Aims and Objectives

At the present time RHDV is still perceived by most RHDV virologists to have emerged once as an epidemic virus in China in 1984. However, the evidence that will be presented herein suggests that this is not the case. Secondly, although it is known that the virus was circulating in rabbits populations throughout Europe, Asia and the Middle East; prior to 1984 the facts that led to the apparent abrupt shift in viral phenotype have never been adequately defined. Thirdly, we have little or no idea how the virus survives between epidemics. Fourthly, the factors that determine the different epidemiological patterns observed for RHDV also need to be explained. Fifthly, we have no idea how RHDV interacts with other viruses such as MYXV. This needs to be investigated. Finally, RHDV has been released as a bio-control agent in Australia and New Zealand but evidence is emerging to suggest it is already losing its effectiveness as a bio-control agent. The data to be presented in this thesis should provide possible solutions to these problems and might enable us to judge the wisdom of releasing large quantities of infectious virus into the environment.

Phylogenetic analysis is a powerful tool with which to study viral origins, evolution, genetic variation, pathogenicity and epidemiology. The work will therefore be based primarily on the construction and analysis of phylogenetic trees. This work will also be supported by serological investigations based primarily on the ELISA test since this procedure is both reliable and adaptable to the study of large number of samples.

This research will benefit from the knowledge and skills of experienced field scientists who have provided the excellent field serum and tissue samples from worldwide sources.

A major advance in our understanding of RHDV and its epidemiology came from the recognition that healthy rabbits may carry viral RNA. This will form the basis of a significant proportion of the research. Viral RNA from both healthy rabbits and also from rabbits sick following infection with RHDV will be sequenced and then compared. These data will then be combined with the information in the databases to construct the most up to date and robust phylogenetic trees with which the evolutionary origins of the epidemic viruses and the apparently harmless viruses may be identified.

There is an important need to understand how RHDV survives within the rabbit populations in the absence of disease. To date few or no genetic changes have been identified between virus isolated from dead rabbits and viral RNA extracted from healthy rabbits (Moss *et al.*, 2002), with the exception of one virus strain designated RCV (Capucci *et al.*, 1996b). Although molecular epidemiological studies are frequently based on partial genomic sequence data, there is a real need for full-length genomic sequences from both healthy rabbits and those that die of RHD. This is particularly important to enable genetic comparisons of strains that exhibit differences in pathogenicity and also provides the opportunity to investigate whether or not genetic exchanges take place between strains of RHDV. Such events might be relevant to our understanding of the basis of RHDV emergence as an epidemic virus.

Finally, the presence of a long-term capture-recapture study provides a unique opportunity to look for evidence of interactions between MYXV and RHDV. This will therefore also be included in the thesis.

It is hoped that the work reported in this thesis will further our understanding of the emergence and evolution of RHDV, as well as the factors involved in shaping the size and intensity of the epidemic outbreaks that are seen today.

There is inevitably some repetition in the introductions of each chapter; this is because each chapter has been written as a manuscript, either published, submitted for publication or in the process of being prepared for publication.

Chapter 2

Materials and Methods

Each chapter contains a separate Materials and Methods section in order to describe the methods specific to that chapter. To avoid repetition the general methods are described in detail below.

2.1 RNA extraction

2.1.1 RNA Extraction from Liver Samples

Viral RNA was extracted using RNAgents kit (Promega) following the manufacturer's instructions, with modifications as described below. Briefly, approximately 0.5g of fresh/frozen rabbit liver (supplied by Dr. Roger Trout and Dr. Brian Boag) or equivalent amount of freeze dried liver (supplied by Dr. Brian Boag) was ground up and 600µl of denaturing solution was added. These suspensions were kept at 4°C for a minimum of 2 days. 60µl of 2M Sodium Acetate (pH 4.0) was added to the processed liver, and the tube was inverted four times to mix the contents throughly. 600µl of the organic layer of Phenol:Chloroform:Isoamyl Alcohol (125:24:1, pH4.7) was added to the liver suspension and the mixture was shaken vigorously for 10 seconds. The mixture was then spun at 10,000 x *g* for 10 minutes to separate the organic and aqueous layers. The aqueous layer (top layer) was extracted and transferred into a fresh tube. 600µl of isopropanol was added to the aqueous layer. The resulting mixture was placed at -20°C for 15 minutes to precipitate the RNA, and then spun at 10,000 x *g* for 10 minutes to pellet the RNA. The supernatant was extracted and 50µl of 80% ethanol was added to the pellet, this was then spun for 1 minute at 10,000 x *g* and then the ethanol was removed. The pellet was left to dry for 5 to 10 minutes depending on its size. When dry, the pellet was resuspended in 50µl of Nuclease free water, and stored at -20°C.

2.1.2 RNA extraction from Sera

100µl aliquots of sera (supplied by Dr. Roger Trout, Dr. Brian Boag and Dr. Horst Schirrmeier) were added to 600µl of denaturing solution and the RNA extraction procedure was then as described above.

2.1.3 RNA extraction from Bone Marrow

Sterile scissors and sterile forceps were used to crack open the bones of rabbits (supplied by Mr Damien Kelly) and approximately 0.1g of bone marrow was extracted. The RNA extraction procedure was then as described above.

2.2 The DNA extraction from Liver

Livers were processed as described above until the Phenol stage, at which 600µl of Phenol:Chloroform:Isoamyl Alcohol (25:24:1, v/v) (Invitrogen) was added to precipitate DNA rather than RNA. The RNA extraction proceedure was then as described above. The resulting DNA suspension was incubated with RNAse A (Gibco) at 10µg ml^{-1} at 37°C for 2 hours.

2.3 Reverse-Transcriptase Polymerase Chain Reaction (RT-PCR)

2.3.1 Nested RT-PCR

Nested-RT-PCR was performed as described in Moss *et al* (2002) with the modifications described below. Reference sequences (Gould *et al.*, 1997, Meyers *et al.*, 1991b, Rasschaert *et al.*, 1995) were used to define the Primers which are described in Table 2.2. A full list of all Primers can be found in Table 2.1 (all primers were used at 100 pmol dilution). Briefly, one microlitre (1µl) of the external reverse primer was incubated with 5µl of RNA extracted as described above, at 95°C

for 2 minutes in a Hybaid thermo-cycler. A buffer containing 4µl of 5x first-strand buffer [250mM Tris-HCl (pH8.3), 375mM KCl, 15mM $MgCl_2$] (Invitrogen), 3µl 6mM dNTP's (Bioline), 1.5µl of 100mM DTT (Invitrogen), 1µl of SuperScript™ II RNase H^- Reverse Transcriptase (Invitrogen) and 1µl of RNasin (Promega) was added to the Primer and DNA and incubated at 42°C for at least 45 minutes. For the PCR step the Biotaq reaction kit was used. Briefly, a reaction buffer was prepared, comprising multiples of: 5µl of the 10x reaction buffer [160mM $(NH_4)_2SO_4$, 670mM Tris-HCl (pH 8.8 at 25°C), 0.1% Tween-20] (Bioline), 2µl of 6mM dNTP's (Bioline), 2µl 50mM $MgCl_2$ (Bioline), 0.5µl BIOTAQ DNA polymerase (Bioline), 40µl of nuclease free water (Promega) and 0.5µl of the required primers. For first round PCR 80µl of this reaction mixture was used and for second round PCR 50µl of the reaction mixture was used. Both first and second round PCR were performed with the primers being the only different ingredients in the mixtures. External primers were used for first round PCR and internal primers used for second round PCR (see Table 2). 50µl of nuclease free Mineral Oil (Sigma) was added to the reaction mixture to prevent evaporation. The entire RT mixture was added to the first round PCR buffer and template denaturation, primer annealing and strand elongation were as follows; 30 cycles of [94°C for 40 seconds, 50°C for 40 seconds and 72°C for 2 minutes] plus a final elongation step of 72°C for 10 minutes. Transfer of 5µl of the first round reaction mixture to the second round reaction mixture was performed and the 30 cycles were repeated. In order to maximise the possibility of amplification from first round PCR, the first round PCR was subjected to an additional 15 cycles using the protocol described above.

Primer Name	Sequence
RHDV 0001F	GTG AAA RTT ATG GCG GYT ATG TCG
RHDV 0382F	RGT YAT YCC CTG TGC TTA CCR GT
RHDV 0557R	GGR YTC TCT TGA TGT CAA YCA GG
RHDV 1057R	TCA AAG CAR CGK TCC GTT GCA CA
RHDV 1217R	GTG AGC TTG CCA GCA CCC TTC A
RHDV 0841F	TTR CTA GTT GGC CTC CAC AA
RHDV 0868F	GCC GTG GAC ACA ACA GTY ATG
RHDV 1432F	AGG TGC ACC CTR CCA TCA TAC AAC CA
RHDV 1503R	TTC CCA AGG ACC ATR GTG TGT
RHDV 2097R	CCA AGA GYT CAC TGT ACT TGT ACT CY
RHDV 2209R	CTG TTT CCA CAT GAG RAG CGC CTG
RHDV 1876F	CAC CCT CGY GCT GGC GCA TTT TA
RHDV 1940F	AAA GYT GGC AAG CCA CCA GRC ATG GYT C
RHDV 2363F	TYC AAC AYC TTT GGC GYA TG
RHDV 2515R	ATG AGC GTG GTY TGT GTG TGG AA
RHDV 3016F	TTR CCC AAG TTC ACY CTT GCW CCR CG
RHDV 3098R	GCR AGR TAC TCC TCT GCA GTC A
RHDV 2872F	AGY TGG AGG CAG ATT GCG CA
RHDV 2926F	AAC CAC CTT GTC AAC CTT GC
RHDV 3419F	CYA GGT CAA GYT GCT CAG A
RHDV 3592R	TAC GCC AGC ACG TCA ATC TT
RHDV 4127R	CAA AGG GYC CGC AAG ASG TTG ACA
RHDV 4163R	TTR CCT GRC GCT CCC CCT TGG TTA GGA A
RHDV 3850F	AGT GGR ACC AGG TAT GCT ARG GTY C
RHDV 3934F	CCA GAC AGT GAC AAG TCA CTC
RHDV 4429F	GTT GGC GTT GAC ATG ACA TC
RHDV 4634R	CAT GGC GTC AAG TAT GGT CAT GG
RHDV 4831F	GTG TAT GCC ATG ACY CCG ATG ATG G
RHDV 4981R	ACC CAC CCR ATG TCC GTG AGC TC
RHDV 5536R	TCC GCG ACK GAC CAA GTR AA
RHDV 5621R	AGC CAG CRT ACA TCT GGC TCA
RHDV 5215F	AGT GTT TAY AGC TAC GAY G
RHDV 5259F	AAG AGA GTC GTC TCG GTA GT
RHDV 5682F	GGT GTG TTT GGT GGG CG
RHDV 5829R	CCA GTT GGA TGG TAC ATG TT
RHDV 6135F	TGG AAC TTG AAT GGC AGC AC
RHDV 6234R	TAC CAAAAC TGA AGC ACG TT
RHDV 6654F	GCC GCT GCA CCT GTG GG
RHDV 6700R	CAC CGG TGC GCC TGA CGA CA
RHDV 7013R	TCA GAC ATA AGA AAA GCC ATT
RHDV 7171R	TTC ATT TGG ATT AAA ACC TAA CC
RHDV 6663F	ACC TGT GGG TAA GAA CAC ACC CA
RHDV 6811F	TAC TCG TCA GCA CTY ATG CCC GGR
RHDV 7437R	ATA GCT TAC TTT AAA CTA TAA ACC YAA

Table 2.1
Showing the oligonucleotides of the primers used to amplify the full-length genome of RHDV. Y corresponds to C/T, R corresponds to A/G, K corresponds to G/T, and W corresponds to A/T. (See table 3.2 page 66 for a detailed description of the combination of primers used to amplify full-length RHDV)

Sequence Name	Author	Accession No.
Spain 89	Parra et al (1993)	Z49271
Czech V351	Gould et al. (1997)	U54938
France 1988	Fischer et al. (1997)	U49726
Germany	Meyers et al. (1991)	M67473
Iowa	Neilan et al. (unpublished)	AF258618
Italy BS89	Rossi (unpublished)	X87607
Mexico 89	Babcock et al. (unpublished)	AF295785
SD	Rasschaert et al. (1995)	Z29514

Table 2.2
Sequences used to design primers used in PCR to amplify the capsid region and the entire genome of RHDV

2.3.2 PCR amplification of DNA

First round and second round PCR were performed as above except first round PCR was performed with 50µl of reaction mixture. 5µl of the extracted DNA (see above) was added to the first round PCR reaction mixture and the protocol was then as above.

2.4 Gel visualisation of DNA

The amplified DNA was visualised under UV light after electrophoresis through a 1% agarose gel in 1 ^{x} TAE buffer (see below) stained with ethidium bromide (EtBr). DNA was loaded into the gel with 2µl of gel loading buffer (see below). The gel was run for 25 minutes at 120V. Amplified DNA was detected by the presence of EtBr, which intercalates with the DNA and exhibits fluorescence under UV light.

Product size was confirmed by comparison to hyperladder I (bioline). Positive samples were then run a second time on a gel and were excised under UV light.

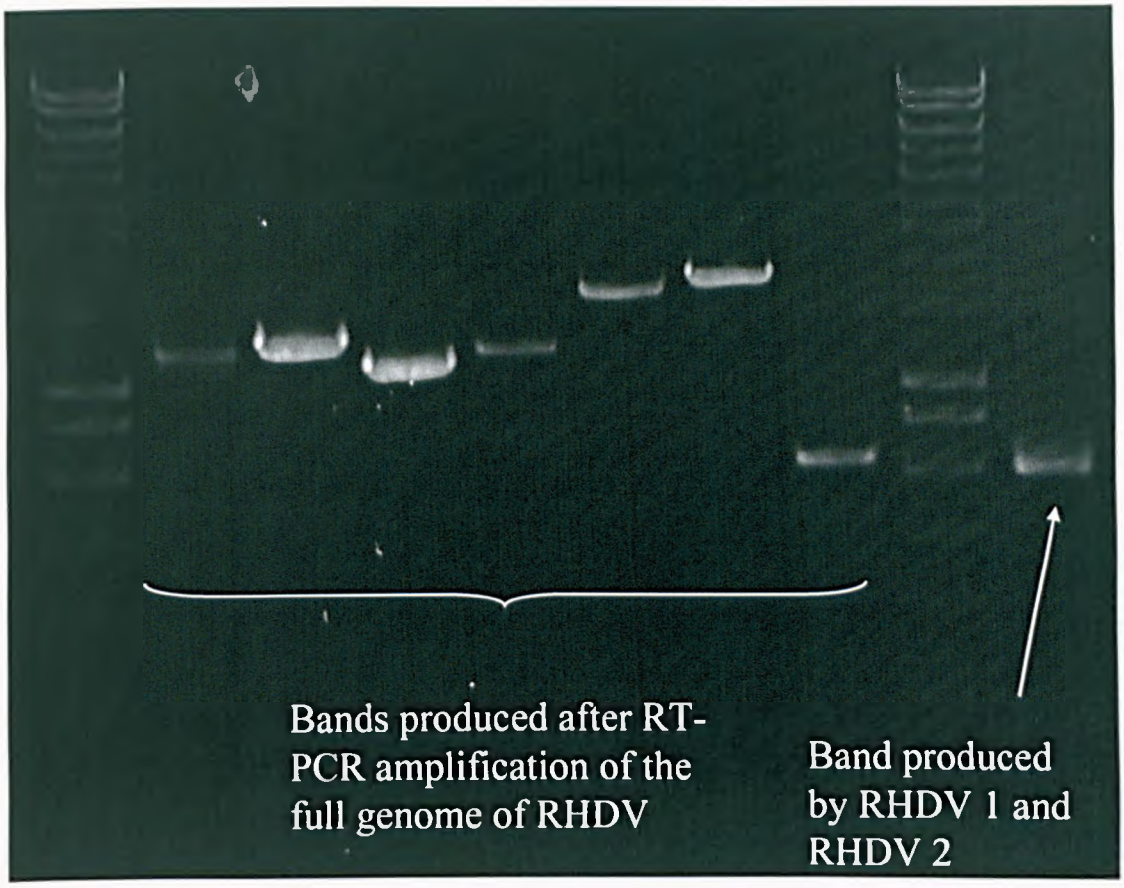

Figure 2.1
Gel picture showing the size of the bands produced when the primers from Table 2.1 were used to amplify the whole genome of RHDV. The band on the far right corresponds to RHDV 1 and RHDV 2 used to test for the presence of viral RNA prior to full genome analysis.

2.5 Gel extraction of DNA

Positive bands of DNA were excised from the gel and processed using the QIAquick® Gel extraction kit (Quiagen) following the manufacturer's instructions and using the buffers provided (unless otherwise stated). The gel slice was weighed and 300ml of QG buffer was added for every 0.1g of agarose gel, and incubated at 50°C for 2-3 minutes, the resulting suspension was briefly vortexed. The QG buffer

resuspends the DNA and contains RNAse activity to give pure DNA. The resulting suspension is then transferred to the QIAquick extraction columns. QIAquick extraction columns contain a silica-gel membrane which absorbs DNA in high salt conditions and releases it in lower salt concentrations. The QG buffer was spun at 14,000 x *g* for 1 min. The flow-through was discarded and then 600µl of the PE buffer, which is used to remove excess salts, was added and spun as before. The column was washed a second time with PE buffer, and then spun twice with the flow-through being discarded after the first round of centrifugation. The spin column was then inserted into a clean collection tube and 50µl of EB buffer, which under the lower salt conditions releases the bound DNA, and spun for 1 minute as above. The DNA was then stored at -20°C.

2.6 Automated DNA Sequencing

Nucleotide sequences of PCR products were determined by ABI prism cycle sequencing, utilising BigDye fluorescently labelled 2',3'-dideoxynucleotides (ddNTPs). During the strand elongation stages of the cycle sequencing reactions, DNA polymerase incorporates standard dNTPs or ddNTPs. When ddNTPs are incorporated the elongation is terminated as the ddNTP lacks a 3'-hydroxyl group, thus preventing the formation of a phosphodiester bridge with the incoming dNTP.

Terminating ddNTPs are 3' labelled with different fluorescent dyes (BigDye) which are used to identify G, A, T or C terminating reactions as they emit different wavelengths of light when excited by a laser. Thus all four colours and consequently all four nucleotide bases can be identified from a single cycle sequencing reaction during capillary electrophoresis.

Cycle reactions were carried out using the BigDye v3.1 Cycle sequencing kit (Applied Biosystems) following the manufacturer's instructions. Reactions comprised 1µl of BigDye v3.1, 1.5µl of the supplied buffer, 2µl of positive or negative sense primer (diluted to 0.5 pmol/µl) (see Table 2), 1µl DNA and 4.5µl of sterile water. The reaction mixture was covered with 20µl of mineral oil (Sigma), and transferred to a Hybaid Thermo Cycler. Template denaturation, primer annealing and strand elongation were as follows; 25 cycles of [96 °C for 30 s; 50 °C for 15 s and 60°C for 4 mins].

Prior to analysis the cycle sequencing products were extracted using Centri-Sep columns (Princeton Separations). The columns were washed with deionised water and spun at 2500rpm for 2 minutes at 4°C. The washed columns were then loaded with Sephadex G-50 Fine Grade Slurry (Sigma) (5g dissolved in 75ml of deionised water). The columns were then spun as above twice discarding the flowthrough after the first round of centrifugation. To collect the reaction mixture the columns were then placed in a clean collection tube. The reaction mixture was extracted from below the mineral oil and placed in the column and then spun as above. Finally a drop of deionised water was added to the column to wash through the DNA and the column was spun again. The product was dried in a Vacuum-dryer and placed at -20°C for storage.

Sequences were run on an ABI 3000 100 machine using capillary electrophoresis and excitation with an argon laser.

2.7 Analysis of Sequences

2.7.1 Phylogenetic Analysis

The products produced by the sequence reactions were assembled as complete sequence data using Pregap4 and Gap4 (Staden Package), subsequent analysis was carried out using translate (GCG package) and transeq (jEmboss). The sequences were aligned using Pileup (GCG, Wisconsin Package) and ClustalX (Thompson *et al.*, 1997). 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 (ML) tree was then estimated using the estimated model, a heuristic search and TBR branch swapping, estimating variable parameters from the data, where necessary (Sullivan *et al.*, 2005). A second tree was drawn using the Neighbour-Joining (N-J) method, with the ML settings. N-J bootstrap support (1000 replicates) was calculated for each tree using the ML settings.

The ML method is a derivation of maximum parsimony (MP), which asks, which of the possible outcomes makes the least number of assumptions? The main difference between ML and MP is that ML uses probability to determine the least number of assumptions, whilst MP uses a stepwise method. The benefits of using the probabilistic method, allows the introduction of a greater number of parameters and therefore fewer assumptions. MODELTEST v3.06 (Posada & Crandall, 1998) was used to estimate the parameters and an iterative approach was used in order to produce the resulting trees. The iterative approach allows us to search probabilistic space with more efficiency, as one of the disadvantages of the ML method is the length of time it takes to test all the probabilities. This method gives a reasonable approximation of the tree.

The N-J method constructs a minimum evolutionary tree by sequentially finding pairs of extant sequences which are connected by only a single interior node. This method does not attempt to cluster the most closely related extant sequences, but rather minimises the length of all internal branches and thus the length of the whole tree (Vandamme, 2003). The benefits of this model are that it is computationally fast and gives a single result.

The ML and N-J methods were used as both use different algorithms and make different assumptions. Thus if the resulting tree from each method correlates it increases the robustness of the tree.

Bootstrapping is the testing of a random subsample of the data. In this method a new bootstrap replicate alignment is constructed by a section of random windows of the original alignment. This process is continued until a new alignment which is the same length as the original is constructed. Each window can be selected any number of times or not at all; this leads to an alignment in which some of the original sequence characters are duplicated and others are omitted. A neighbour-joining phylogenetic tree of the bootstrap alignment is then constructed using the Maximum likelihood settings. This process is repeated 1000 times producing percentage support for each of the branch nodes. In this study 1000 bootstrap replicates were performed for each dataset. Branches that were supported by bootstrap confidence intervals of less than 75% were treated with caution (Felsenstein, 1985).

2.7.2 Basic Local Alignment Search Tool (BLAST)

To determine the sequences flanking remnants of RHDV VP60 gene isolated from the DNA of rabbits, searches were conducted of the mammalian sequence

databases utilising the Basic Local Alignment Search Tool (BLAST) (Altschul *et al.*, 1990). Blast is a heuristic programme, which performs local alignments as opposed to global alignments. This offers the advantage of being able to find more similarities in homologous sequence accessions which contain large scale indels that interrupt similarity to the sequence under investigation.

When scoring sequences within a database, the BLAST programme builds a position specific matrix for high scoring accessions. This is achieved by breaking the sequence under investigation into short fragments and then searching the (nucleotide) sequence databases for identical or close matches. Following the determination of an equivalent sequence (hit), a local alignment is generated by extending the closely matching sequence in both directions. The quality of each alignment is then statistically examined to provide an indication of similarity to the sequence under investigation. This is achieved by calculating the probability of the alignment arising by chance, which is referred to as the expected value (E – value). An alignment with low E- value (close to zero) is less likely to have occurred by chance and has a greater statistical significance than an alignment that has a higher E-value. For example, a sequence alignment with an E-value of 0.05 indicates that this similarity has a probability of 5 in 100 (1 in 20) of occurring by chance alone. Alignment pairs that have low E-values are referred to as high scoring segment pairs (HSPs). Sequence accessions that contain HSPs are presented at the top of the BLAST result output. A further advantage of the BLAST is that within a results output the positions of HSPs within a sequence accession are listed which facilitates further sequence analysis. However, a disadvantage is that only regions of high homology to the sequence under investigation are listed, the internal or flanking

regions are not retained. To analyse these further, high scoring accessions must be examined individually.

2.8 Enzyme-linked immunosorbent assay

2.8.1 ELISA test for rabbit IgG antibodies against RHDV

Rabbit blood samples were collected as described in White *et al.* (2004), or from fresh shot rabbits (supplied by Dr. Roger Trout and Dr. Peter White). Blood samples were spun at 10,000 x g for 10 mins to collect sera which were then used in the ELISA tests. ELISA tests were carried out as described in Moss *et al.* (2002). Briefly, two 96 well plates (Dynex technologies) were coated with the recombinant baculovirus protein (Marin *et al.*, 1995) and the control baculovirus protein, to eliminate any background readings due to the baculovirus protein. The two plates were labelled Ag and Control, the Ag plate was coated with 1µl of the recombinant baculovirus protein in 25ml of 1x coating buffer (1:25,000). The control plate was coated with 7µl of the protein control in 11.2ml of 1x coating buffer (1:1600). 100µl of the coating buffer was added to each well and the plates were sealed with a plastic coat and placed at 4°C overnight.

The plates were washed three times with PBS/Tween (0.1%). A blocking solution of 0.4g bovine albumin (Sigma) in 40ml of PBS/Tween (0.1%) was prepared. 180µl of the blocking solution was added to each well, and the plates were wrapped in plastic before being incubated at 37°C for 1 hour in a humidified box. The plates were then washed three times with PBS/Tween (0.1%). 100µl of PBS/Tween (0.1%) was added to each well with an extra 80µl being added to the top row and to the negative control.

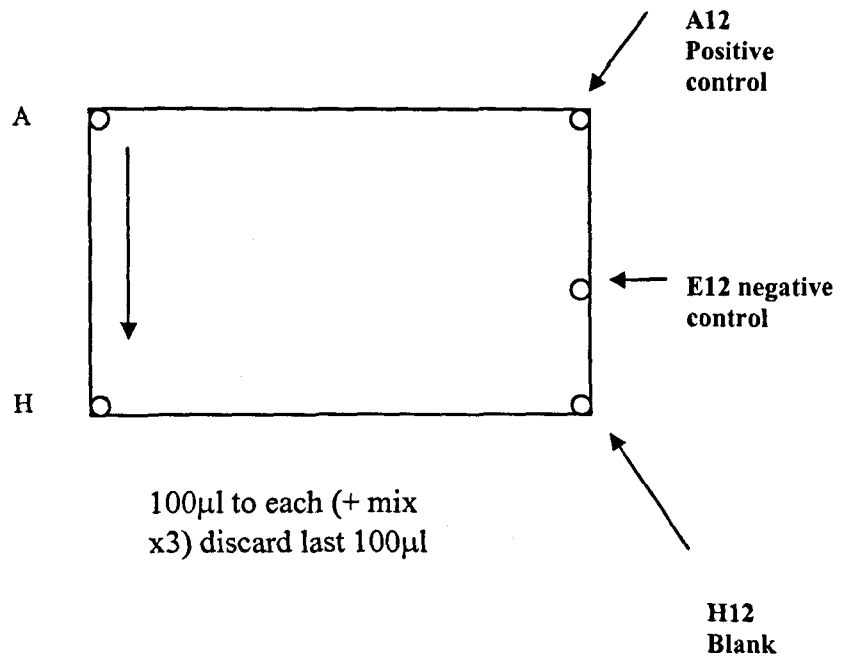

Figure 2.2
Showing the layout for the ELISA test for RHDV-specific antibodies

Sera were added to row A 1-11, with a positive control added to A12 and a negative control to E12. The contents of row A were mixed three times and then 100µl transferred to the next well, where the process was repeated, 100µl was discarded from row H. H12 was kept blank as a further control. The plates were then incubated at 37°C in a humidified box for 2 hours.

The plates were then washed three times with PBS/Tween (0.1%) leaving the last wash in the plates for a little longer. 4µl of the conjugate antibody, goat anti-rabbit immunoglobulins (HRP conjugated) (DAKO), was added to 20ml PBS/Tween (0.1%) and 100µl of this mixture was added to each well, and then incubated in a humidified box for 1 hour.

The plates were then washed four times with PBS/Tween (0.1%). The substrate Fast™ o-phenylenediamine dihydrochloride table sets (Sigma) was used following the manufacturer's instructions. Four tablets were dissolved in 40ml of deionised water and 190µl of the resulting mixture was added to each well. The plates were then placed in the dark for 30 minutes. The reaction was stopped by adding 50µl of 2M HCl to each well. The plates were then read at 492nm on a Titertek Multiskan Plus. The titre of the antibody was determined as the inverse of the dilution prior to the dilution when the difference between the control and antigen plate was less than 0.1. The difference was calculated by subtracting the control readings from the antigen readings.

2.8.2 ELISA test for rabbit IgM antibodies against RHDV

The procedure followed was that described above except for the conjugate antibody stage. Here 1µl of conjugate, goat-anti rabbit IgM (HRP-conjugated) (Bethyl Laboratories) was added to 40ml of PBS/Tween (0.1%) and the procedure then followed as above.

2.8.3 ELISA test for rabbit IgG antibodies against Myxoma Virus (MYXV)

Purified MYXV was used as the substrate for these ELISA's. 100µl of purified MXYV (details below) was diluted in 4ml of PBS. 100µl of the coating solution was added to each microtitre assay plate (Dynex technologies), the plates were then wrapped in plastic and left overnight in the fridge. The procedure was followed as above except, row G was used as a positive control and row H was used as a negative control; H12 was left blank as above.

2.9 Preparation of MYXV stocks for use in ELISA tests

RK13 cells were grown in L-15 media (Gibco) with 10% Fetal bovine serum (FBS) (Gibco) and 1% Pencillin/Streptomycin (P/S) (Gibco). The cells were infected with MYXV (Lausanne strain) in MEM (Gibco) with 3% FBS and 1% P/S. Cells infected with 1ml of virus (titre 3 x 10^6) for 3 days and showing cytopathic effects were scraped from the flasks using a disposable silicone scraper (Falcon), sedimented by low-speed centrifugation and washed once in TL20 buffer (Gelfi *et al.*, 1999). The sedimented pellet of cells was resuspended in TL20 buffer and placed overnight at -70°C. The suspension was homogenized using a Dounce homogenizer and clarified by centrifugation at 1,200 x *g* (2,500 rpm) for 10mins.

The supernatant fluid was collected and layered onto a 36% (w/v) sucrose cushion and the virus antigen was sedimented by centrifugation at 200,000 x *g* (30,000 rpm, SW41.14 rotor) for 2 hours. The resulting pellet was resuspended in TL20 buffer.

Appendix 1: Buffers used

TAE buffer (x50)

242g Tris (AnalaR)

57.1ml Acetic Acid (AnalaR)

19g EDTA (AnalaR)

Made up to 1 litre with deionised water

Gel Loading Buffer

1.5ml Glycerol (AnalaR)

1.5ml 1x TAE

Bromophenol Blue (BDH Ltd)

Coating Buffer (x10)

1.59g Sodium Carbonate (AnalaR)

2.93g Sodium Hydrogen Carbonate (AnalaR)

Made up to 100ml with deionised water

PBS/Tween (0.1%)

500ml PBS (Oxoid)

0.5ml Tween 20 (Difco)

TL20

10ml Tris pH8.6 9 (see below)

0.19g EDTA (AnalaR)

4.4g NaCl (AnalaR)

Made up to 500ml using sterile water and autoclaved.

1M Tris pH8.6

60.6g Tris (AnalaR)

dissolved in 250ml sterile water

Calibrated to pH8.6 using 2M HCl (MERCK)

Made up to 500ml using sterile water and then autoclaved

36% Sucrose Solution

36g sucrose (AnalaR)

made up to 100ml with sterile water

Chapter 3

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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3.1 Summary

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 we have investigated them 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 closely related to the released Czech strain of RHDV, although the sequence from one rabbit was most closely related 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 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.

3.2 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 recognised 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 etiological agent was identified as a calicivirus (Ohlinger *et al.*, 1990), a positive single stranded RNA virus, which is antigenically related to *European brown hare syndrome virus* (EBHSV) (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 biological control agent in Australia, where the rabbit population was devastating the indigenous flora and fauna (Asgari *et al.*, 1999, Sandell, 2002a). In 1995 a series of field trials was initiated on Wardang Island, 5 kilometres off the coast of Port Victoria, on the Yorke Peninsula on the 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 of Australia (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 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 deliberately introduced illegally into New Zealand in 1997 (Motha & Clark, 1998, O'Keefe *et al.*, 1998).

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 sub-lethal 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 inadvertently introduced 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 following 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, four years after its introduction to 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.

3.3 Materials and Methods

3.3.1. Rabbit material

Apparently healthy rabbits were shot at 8 sites in Central Otago all in the vicinity of Alexandra. 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 hours) until processed, therefore, no blood samples were obtained. The liver and bone marrow of all the rabbits were extracted and freeze dried. Table 3.1 shows the distances between the sites and Fig. 3.1 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 recently been published (Zheng *et al.* 2002).

	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

Table 3.1
Straight-line distance between sites in kilometres

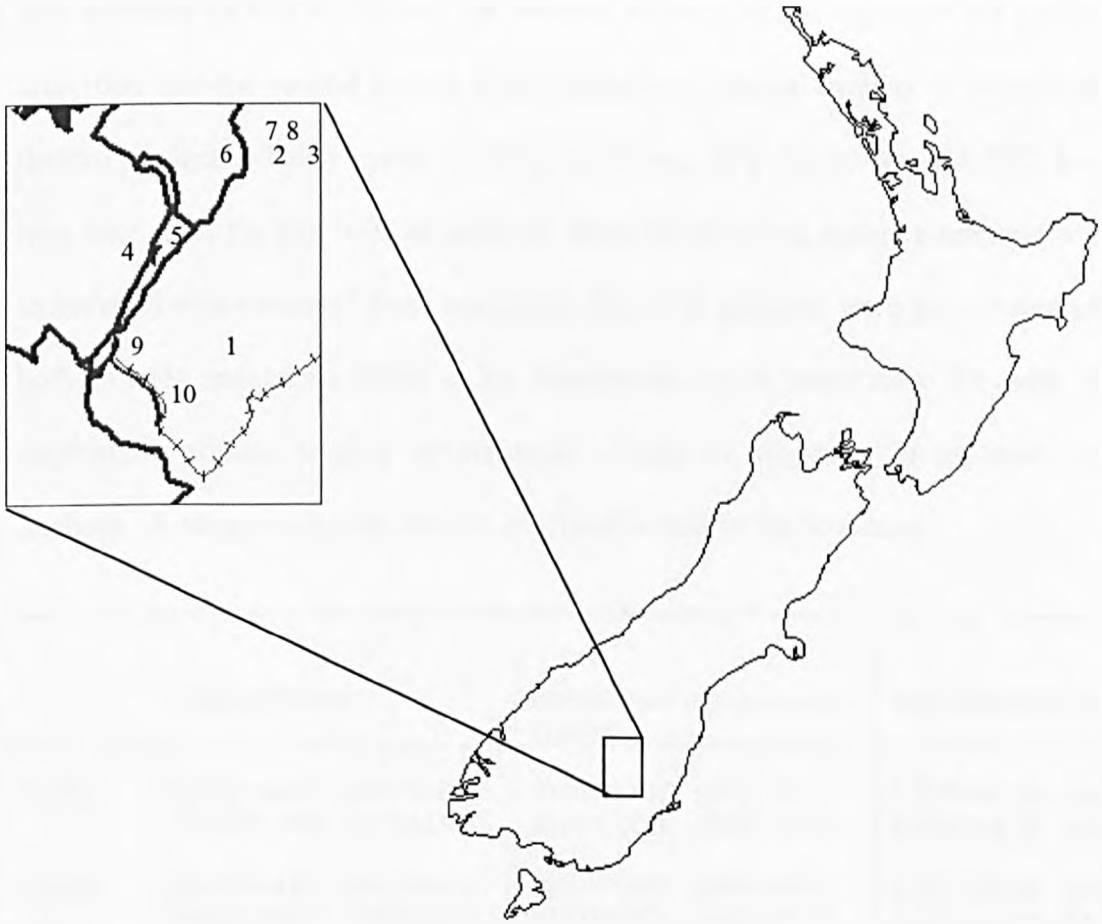


Figure 3.1
Geographic distribution of the sites from which samples were obtained in Central Otago, New Zealand

3.3.2 RT-PCR Sequencing

Viral RNA was extracted from liver samples using RNAgents kit (Promega). Primers for RT-PCR were designed from known sequence based on full genome sequences (Gould *et al.*, 1997, Meyers *et al.*, 1991b, Rasschaert *et al.*, 1995), and are listed in Table 3.2. 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, the

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

	External Primers	Internal Primers/Sequencing Primers	Extra Sequencing Primers
0-1KB	RHDV0001F (0001-0024) RHDV1217R (1217-1238)	RHDV0001F (0001-0024) RHDV1057R (1057-1079)	RHDV0607R (0607-0629) RHDV0382F (0382-0404)
1-2KB	RHDV0841F (0841-0860) RHDV2209R (2209-2232)	RHDV0868F (0868-0888) RHDV2097R (2097-2122)	RHDV1503R (1503-1523) RHDV1432F (1432-1457)
2-3KB	RHDV1876F (1876-1898) RHDV3098R (3098-3119)	RHDV1940F (1940- 1967) RHDV3016R (3016-3041)	RHDV2515R (2515-2537) RHDV2363F (2363-2382)
3-4KB	RHDV2872F (2872-2891) RHDV4163R (4163-4190)	RHDV2926F (2926-2945) RHDV4127R (4127-4150)	RHDV3592R (3592-3611) RHDV3419F (3419-3437)
4-5.5KB	RHDV3850F (3850-3874) RHDV5621R (5621-2641)	RHDV3934F (3934-3954) RHDV5536R (5536-5555)	RHDV4634R (4634-4656) RHDV4429F (4429-4448) RHDV4981R (4981-5003) RHDV4831F (4831-4855)
5.5-7.1KB	RHDV2515F (5215-5238) RHDV7171R (7171-7191)	RHDV5259F (5259-5278) RHDV7023R (7023-7043)	RHDV5829R (5829-5848) RHDV5682F (5682-5698) RHDV6234R (6234-6253) RHDV6135F (6135-6154) RHDV6700R (6700-6719) RHDV6654F (6654-6670)
6.8-7.4 KB	RHDV6663F (6663F-6685) RHDV7437R (7411-7437)	RHDV6811F (6811-6833) RHDV7437R (4711-7437)	

Table 3.2
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. The sequencing primers were used in addition to the internal primers to give approximately 500bp of sequence for assembly and analysis

3.3.3 Phylogenetic Analysis

The 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+Γ 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.

3.3.4 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. One hundred micro-litres of each suspension were injected by the intramuscular route into individual white Swiss rabbits. The rabbit sera were monitored using ELISA tests to detect the presence of antibodies and the livers were subsequently examined using nested RT-PCR as described above.

3.3.5 ELISA Tests

3.3.5.1 IgG Detection

An optimised 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 hr at 37°C. The plates were washed and a 1:1000 dilution of polyvalent goat anti-rabbit serum conjugated with horse-radish peroxidase (Sigma-Aldrich) was added for one hour 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.

3.3.5.2 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 horse-radish peroxidase (Bethyl Laboratories) was used.

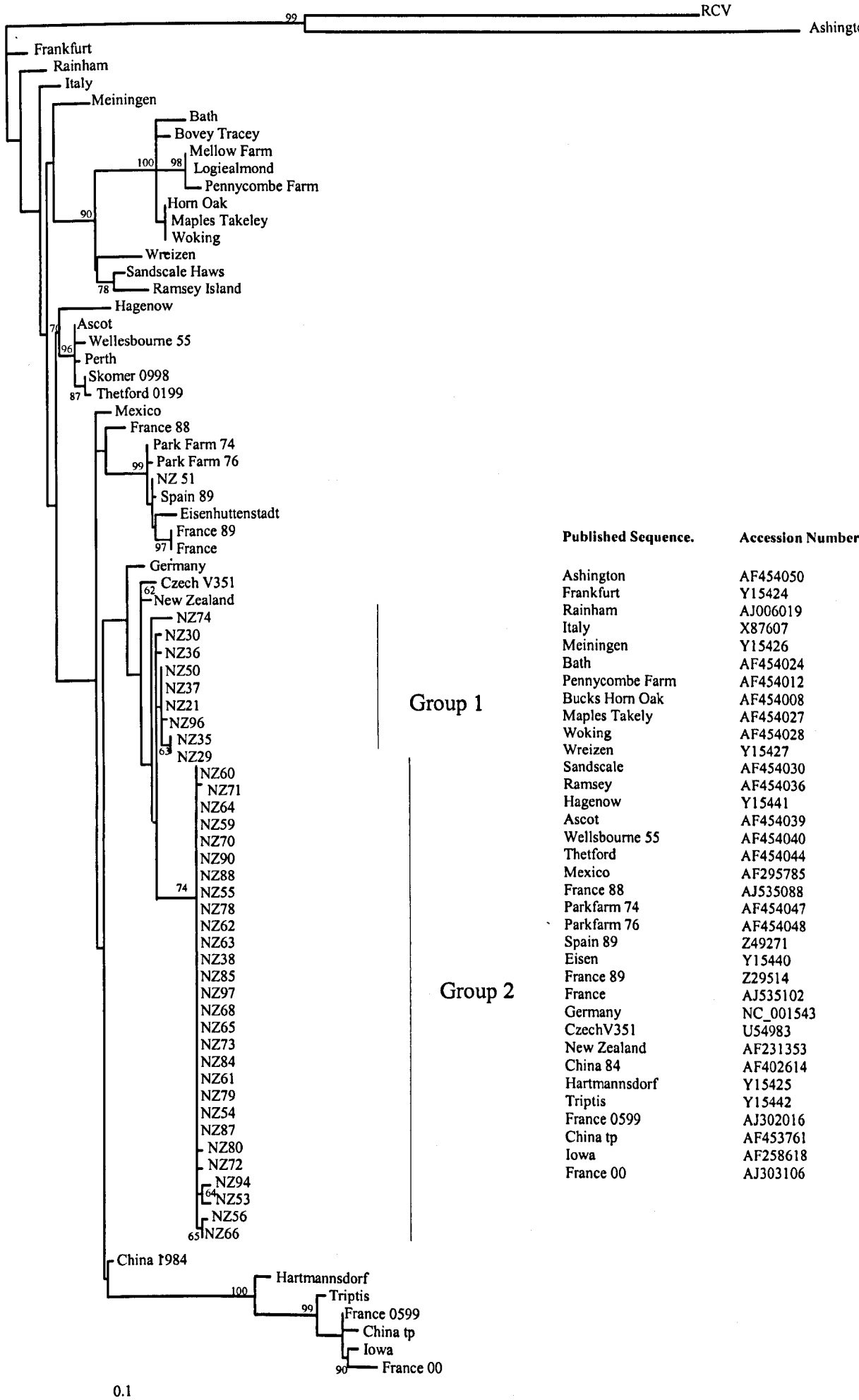
3.4 Results

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

Seventy-six liver samples obtained from 8 different sites in Central Otago, New Zealand were analysed by nested RT-PCR sequencing, an approach that targeted the viral capsid gene using RHDV 5682F and RHDV7032R for the first round of PCR, and RHDV 6135F and RHDV6700R for the 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 (527bp). 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 ClustalX (Thompson *et al.*, 1997). The maximum genetic variability was 2.6% 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. 3.2) 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 closely related to the Czech strain V351 that was deliberately introduced into New Zealand in 1997. However the sequences of these samples formed two distinct groups. One group clustered close to the deliberately introduced Czech strain and the New Zealand sample sequenced in the first two years after release. The second group was similar to but distinct from the Czech strain. Within each group very little variability was detected and both groups appear to have varied equally although they remain distinct. At 5 of the 8 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 know from which sub-population the samples came. As can be seen in Figure 3.2, one sample (NZ51) amplified from rabbit liver (Site 6) was positioned within a clade containing European viruses (Fig. 3.2) and most closely related 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 therefore amplified a region of the NZ51 genome outside the capsid genome (0-1KB) to confirm that this Spanish related



Published Sequence.	Accession Number
Ashington	AF454050
Frankfurt	Y15424
Rainham	AJ006019
Italy	X87607
Meiningen	Y15426
Bath	AF454024
Pennycombe Farm	AF454012
Bucks Horn Oak	AF454008
Maples Takely	AF454027
Woking	AF454028
Wreizen	Y15427
Sandscale	AF454030
Ramsey	AF454036
Hagenow	Y15441
Ascot	AF454039
Wellsbourne 55	AF454040
Thetford	AF454044
Mexico	AF295785
France 88	AJ535088
Parkfarm 74	AF454047
Parkfarm 76	AF454048
Spain 89	Z49271
Eisen	Y15440
France 89	Z29514
France	AJ535102
Germany	NC_001543
CzechV351	U54983
New Zealand	AF231353
China 84	AF402614
Hartmannsdorf	Y15425
Triptis	Y15442
France 0599	AJ302016
China tp	AF453761
Iowa	AF258618
France 00	AJ303106

Figure 3.2 (Figure on preceding page)
Phylogenetic analysis using partial capsid sequence (see Methods) for 78 strains of RHDV. Maximum Likelihood phylogeny was calculated using PAUP* version 4.0b8. The optimal model to use with the data (K80+Γ) was determined using MODELTEST 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.

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sequence was not a laboratory contaminant. It is also important to emphasise that at the time of collection of these rabbits, there were no signs of fatal epidemic RHDV.

3.4.2 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, utilising all the primers defined in Table 3.2. These sequences were then compared against the complete genome sequence of the Czech strain. The 527bp region and the full-length sequence of NZ54 showed 97.5% and 97.6 % nucleotide identity with respect to the CzechV351 strain. The amino acid sequence was 98.6% homologous with the Czech strain. The 527bp 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 0-2kb 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 successfully sequenced with similar results.

3.4.3 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 known positive liver samples. None of the rabbits showed clinical signs of infection. Sera were collected before inoculation and at the termination of the experiment. The 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.

3.5 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 previously been present 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 sub-clinical

virus infection. This is not without precedent since a non-virulent strain of RHDV in domestic rabbits in Italy has been identified and characterised (Capucci *et al.*, 1996b), and RHDV-specific RNA has recently been detected 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 recognised (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), that resulted following the deliberate release of the virus by the farmers (Cooke, 2002).

We found RNA closely related to two supposedly virulent strains of RHDV in the livers of healthy rabbits in New Zealand, viz., the Czech V351 and 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 recognised lethal viruses, i.e. viruses obtained from dead or dying rabbits have yielded PCR products on first round PCR amplification (unpublished data). It could therefore be argued this sample represents a virus more closely related 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 closely related to the Czech strain, known to have been used by the farmers who deliberately spread the virus. However the 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 three years after the virus was introduced (O'Keefe *et al.*, 1998). The second group was more distantly related, and it appeared that the divergence point of the virus occurred before the virus was introduced into New Zealand (Fig. 3.2). 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. The two groups therefore 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 strongly argues 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 inadvertently introduced 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 previously been recognised 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 sub-clinical persistent and/or latent infections. It has been suggested that the unorthodox and unregulated introduction of the virus could have caused a sub-lethal 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 thus far been tested 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 shot showed no evidence of any RHDV epidemic at the time of collection [personal observation-BB]. Therefore the rabbits have either been carrying the viral RNA since the last epidemic, or as was previously suggested, there is a virus circulating that does not cause severe morbidity and mortality (Capucci *et al*, 1998, Chasey *et al*, 1997a, Rodak *et al*, 1990, Trout *et al*, 1997b). 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) closely associated 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 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 they 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.

Chapter 4

Can phylogenetic analysis unravel the paradox of the emergence of Rabbit haemorrhagic disease virus in 1984?

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4.1 Summary

During the past 50 years two viruses have decimated wild rabbit populations worldwide. When thousands of domestic rabbits unexpectedly died in China in 1984, it was assumed that this was a new disease agent. Questions were asked. Was it the new myxomatosis? How did it arise? Would it disperse outside China? Was it the perfect biological agent to control wild rabbit populations and thereby reduce damage to agriculture and forestry? Would its impact on rabbits be so severe that predator species would become extinct, hunters have nothing to hunt and important conservation habitats, maintained by rabbit grazing, become overgrown? Some of these questions have subsequently been answered but our studies on the virology have revealed a paradox. If we are to believe the evidence, it seems that existing widely dispersed strains of the aetiological agent, Rabbit haemorrhagic disease virus (RHDV), must have spontaneously altered their virulence almost immediately after the appearance of the Chinese epidemic strain in 1984! This review uses phylogenetic analysis to present a rational explanation for the emergence and evolution of RHDV and considers the differing impact of RHDV on rabbit populations worldwide.

4.2 Introduction

The wild European rabbit (*Oryctolagus cuniculus*) in Britain contributes significantly to the food supply of a variety of predators, and its grazing is largely responsible for the maintenance of key conservation habitats, such as calcareous grassland and dune slacks as well as food plants for a number of rare vertebrate and invertebrate species. However, rabbits also damage agricultural and forestry enterprises by over-grazing, and their burrowing activities also impact on the infrastructure of embankments and foundations (Thompson, 1994, Trout, 2003). While small scale population fluctuations are common, land managers strive to maintain acceptable levels. However, this delicate balance was seriously disturbed when myxoma virus (MYXV) was introduced into Britain in 1953. This led to a major collapse in rabbit population density with only a gradual recovery during the following thirty years (Tapper, 1992, Thompson & Worden, 1956, Trout *et al.*, 1986). It is believed that recovery was partly assisted by the appearance of MYXV strains with reduced virulence (Fenner & Marshall, 1955, Hudson & Mansi, 1955) and partly by the development in rabbits of genetic resistance to MYXV (Ross & Sanders, 1977, Ross & Sanders, 1984) which increased as population density recovered. There was also evidence that the presence of more virulent strains of MYXV increased (Ross & Sanders, 1987). Nevertheless, myxomatosis still had a controlling effect on rabbit populations, (Fenner & Fantini, 1999, Trout *et al.*, 1992).

The impact on the British countryside was dramatic and quite complex. Apart from the reduced prey source other changes include parasite and disease incidence, changes in microclimate from cessation of scratching and burrowing, to floral and invertebrate influences (Sumption & Flowerdew, 1985, Thompson, 1994). Conservation groups struggled to avoid the scrub invasion and choking of rare

habitats and the decline and extinction of rare species caused by the cessation of grazing and burrowing, and hunters were denied a previously common quarry.

4.2.1 Rabbit haemorrhagic disease virus

Rabbit haemorrhagic disease virus (RHDV) was first isolated in 1984, when a previously unknown fatal illness swept through the domestic rabbit population of China, killing an estimated 14 million rabbits (Xu, 1991). The epidemic originated in Wuxi City in Jiangsu province and dispersed rapidly across China, covering 50,000km^2 in nine months (Xu, 1991). For some years the aetiological agent was thought to be a parvovirus. However in 1990 it was identified as a calicivirus (Ohlinger *et al.*, 1990, Ohlinger & Thiel, 1991, Parra & Prieto, 1990). RHDV is a single-stranded positive-sense RNA virus with a genome length of 7.5kb. It has two open–reading frames (ORF), with ORF1 encoding a polyprotein of 257kDa, which is post-translationally cleaved into nine proteins (Konig *et al.*, 1998, Meyers *et al.*, 2000), including the capsid protein. ORF2 is frame-shifted with respect to ORF1 and encodes a structural protein of 10kDa (Meyers, 2003).

From its presumed origin in China, the disease appeared to spread throughout domestic and subsequently wild rabbit populations in Asia and Europe. It is believed that the transportation of infected rabbits and/or infected rabbit products contributed significantly to this rapid dispersal. The first epidemics in domestic rabbits in Europe were reported to have caused mortality rates of about 80%, possibly reflecting the fact that juveniles up to 2 months of age are resistant to infection. In contrast, the mortality rate in wild rabbits in France was nearer to 50% (Marchandeau *et al.*, 1998).

The first recorded epidemic in wild rabbits in Spain in 1988 (Cooke, 2002, Villafuerte *et al.*, 1994), subsequently appeared to spread throughout the wild populations of most European countries, and by 1992 rabbit haemorrhagic disease (RHD) was recorded for the first time in Britain (Chasey, 1997, Fuller *et al.*, 1993). As a novel and notifiable disease between 1992 and 1996, MAFF followed the pattern of outbreaks in domestic and wild populations (Trout, *et al.*, 1997a). Wild rabbit populations were reported as in major decline in some localities from 1994. One site in Devon was being counted at the time of the first outbreak and the population collapsed and has remained low ever since. Ten years later dune slacks still require cutting in an attempt to prevent extinction of rare plants. A nearby estate has reduced its use of bullets from c.22000 to 2000 per year. Outbreaks in the UK were scattered, slowly coalescing over the years. The most significant reductions appeared to be in the south west but the situation has remained unclear. Several independent surveys report that rabbit numbers have declined nationally by about 20% since the mid 1990s when virulent outbreaks were reported throughout Britain (Battersby, 2005), nothing like the myxomatosis epidemic 40 years before. The longer term impact on a sample of 24 populations in England seemed to be influenced by the severity of the original outbreak (Fig. 4.1).

The high virulence of RHDV for domestic rabbits led to it being investigated as a potential bio-control agent in Australia and New Zealand, where the European rabbit is a significant pest (Asgari *et al.*, 1999, Sandell, 2002b). In 1995 a series of field trials was carried out on Wardang Island, a few miles off the southern coast of Australia. However, during the trials, the virus escaped to the mainland, and spread rapidly across southern Australia killing up to 98% of the wild rabbits in some regions, possibly aided and abetted by mechanical transmission due to biting insects.

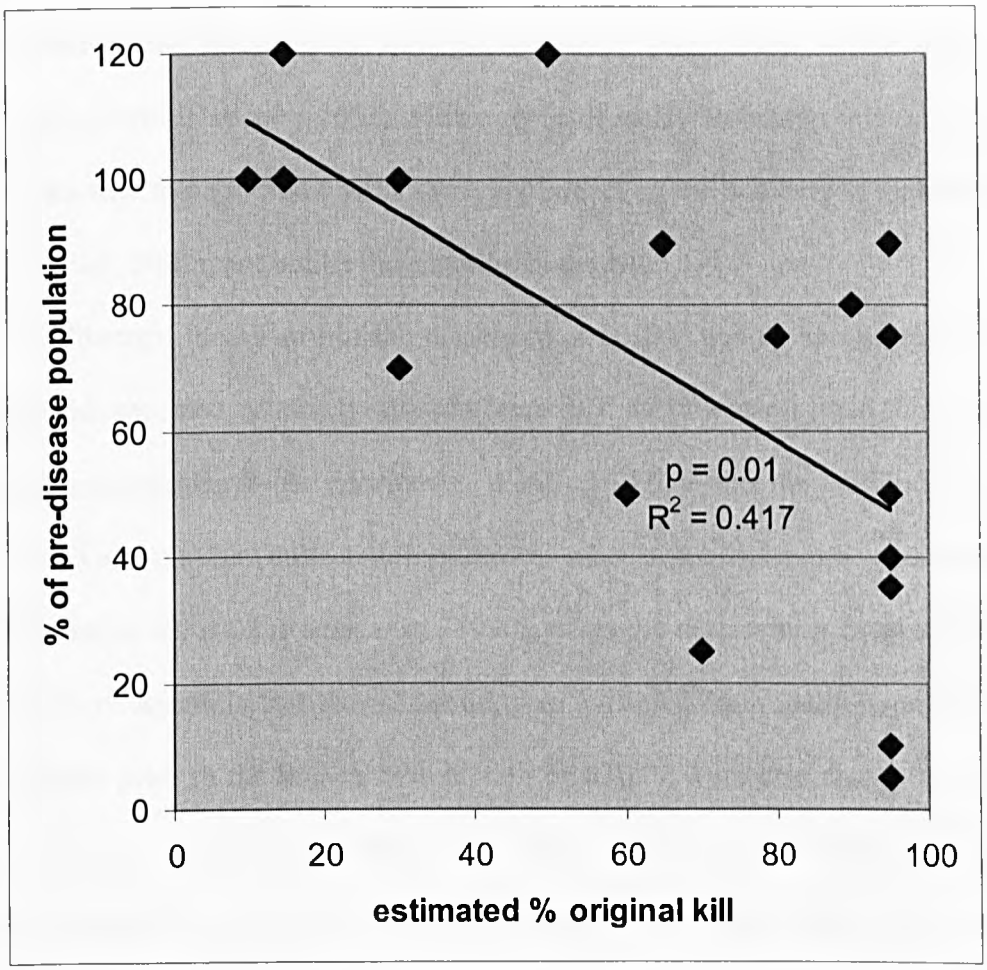

Figure 4.1 Estimates of immediate change in rabbit populations from 24 sites in England and subsequent reassessment 5-7 years later. The linear regression is significant; regressions of samples from 1994, 1995 and 1996 follow the same pattern. The data indicate that heavy initial reductions tend to be followed by a poor recovery whilst small initial reductions are followed by population recovery. Courtesy of R. Trout

Major recovery of the vegetation and the reappearance of seedlings of declining species became widespread in drier areas of Australia, but the disease was less successful in wetter areas. Then in 1997, the virus was introduced illegally into New Zealand (Motha & Clark, 1998), and deliberately spread by the farmers, initially, though not universally, with the same devastating impact on rabbits as had previously been observed in Australia. However, RHDV has now apparently

established a less lethal status amongst the rabbit populations within these two countries (Henzell *et al.*, 2002, Mutze *et al.*, 2002, Robinson *et al.*, 2002a). Outbreaks tend to be sporadic with a varying impact on the numbers of rabbits killed (Parkes *et al.*, 2002), not unlike the situation in the UK.

Although, the history of the discovery of RHDV and its spread through the Old World appeared relatively straightforward, it is now clear that some of the original assumptions were incorrect. Firstly, RHDV-specific antibodies were detected in archival sera collected from healthy rabbits and frozen many years before 1984 (Moss *et al.*, 2002, Rodak *et al.*, 1990), when the disease was first recognized. Moreover, in Australia and New Zealand, sera collected from many populations of wild rabbits prior to the known introduction of RHDV were also shown to contain RHDV-specific antibodies (Chasey *et al.*, 1997a, Moss *et al.*, 2002, Nagesha *et al.*, 2000, O'Keefe *et al.*, 1999, Robinson *et al.*, 2002a, Rodak *et al.*, 1990). This implies that RHDV circulated silently amongst domestic and wild rabbit populations in Europe, Asia, Australia and New Zealand prior to the first recognised epidemics. Subsequent analysis of the archival sera using RT-PCR confirmed the presence of RHDV-specific RNA in UK samples but with no associated disease (Moss *et al.*, 2002). The sequence of this viral RNA was remarkably similar to that obtained more than 40 years later in samples taken from wild and domestic rabbits that died following infection with RHDV. Secondly, genomic-length viral RNA was recently detected in livers from healthy rabbits collected in New Zealand (Forrester *et al.*, 2003, Zheng *et al.*, 2002). This RNA showed a high degree of sequence similarity to the deliberately released virulent Czech bio-control agent. However, it is important to note that infectious virus has never been isolated from these healthy rabbits, only RNA. Thirdly, the epidemiological characteristics of RHDV in wild rabbits vary in

different regions of the Old World. In Spain, although seasonal cycles do exist, high rabbit mortality due to RHDV can be observed throughout the year. In Spain the decline of rabbits exacerbated the precarious position of the obligate rabbit predators, Spanish Lynx (*Felix pardena*) and Spanish Imperial Eagle (*Aquila adalberti*) by virtually eliminating their food supply (Angulo & Cooke, 2002). Frantic efforts are in progress to encourage the rabbit population by large scale translocations, habitat modification, and even the experimental production of a live Mxyoma-RHD GMO vaccine (Torres *et al.*, 2001). In contrast, in the UK the epidemiological pattern is unpredictable and apparently quite random. For example, it is not unknown for two adjacent rabbit populations to show independent responses in the face of RHDV. One population may crash whilst at the same time the other may remain free of the disease, or register only a small impact. A similar pattern has also been observed in France. Meanwhile the same virus has been found simultaneously at a distance of up to 150km as though it had been transported directly to the distant site – perhaps by human or insect vectors. These epidemiological patterns have also been reported in Australia, where in the semi-arid regions the epidemiology is similar to that found in Spain but in the wetter coastland areas it resembles more closely the pattern seen in the UK and New Zealand (Henzell *et al.*, 2002, Mutze *et al.*, 2002).

During the early and mid 1990's it was observed that many healthy rabbits possessed antibodies that protected them from experimental challenge with RHD (Chasey *et al.*, 1997a, Trout *et al.*, 1997a). One plausible explanation was that a non-pathogenic virus circulated within these wild rabbit populations producing protective antibodies (Trout *et al.*, 1997b, White *et al.*, 2001). Added weight was given to this with the discovery of a distinct non-pathogenic calicivirus, Rabbit Calicivirus (RCV) in Italian domestic rabbits, similar to RHDV and able to protect against RHDV

(Capucci *et al.*, 1996b). Whilst this hypothesis was generally accepted, recent virological studies have cast doubt on it. Here we use phylogenetic methods to analyse the evolution and ecology of RHDV in the context of its origin, dispersion, virulence for rabbits and its use as a bio-control agent.

4.3 Materials and Methods

4.3.1 RT-PCR sequencing

Rabbit bones, from animals presumed to have died following infection with RHDV were collected from five sites in Ireland, Ballygawley, Castlewellan, Cullybacky, Letterkenny and Smithboro'. Viral RNA was extracted from the bone marrow using the RNAgents kit (Promega) following the manufacturer's' instructions. Primers for RT-PCR were designed from known sequence based on the capsid protein VP60 (Boga *et al.*, 1994, Capucci *et al.*, 1996a, Le Gall *et al.*, 1998, Le Gall *et al.*, 1992). We sequenced the complete capsid gene; the primers used are described in Table 4.1. First strand cDNA synthesis was performed using Superscript II reverse transcriptase (Invitrogen) with RHDV7171 primer. A nested PCR was used to amplify the DNA, the first reaction (RT-PCR) utilised primers RHDV5215 and RHDV7171, and the second (nested PCR) utilised primers RHDV5259 and RHDV7023 to produce a product of 1750 bp. Thirty cycles of 94°C for 40 sec., 50°C for 40 sec. and 72°C for 1 min. were used for both sets of primers. These PCR products were gel purified and both strands sequenced using a PE Biosystems cycle sequencing kit using the primers described in Table 4.1 to give 1750 nucleotides of sequence for analysis.

Rabbit livers were also obtained from healthy animals collected on Coll Island, Scotland. RNA was isolated from the liver and amplified using the RNA extraction and PCR protocols described above.

	External Primers	Internal Primers/Sequencing Primers	Extra Sequencing Primers
	RHDV5215F (5215-5238) RHDV7171R (7171-7191)	RHDV5259F (5259-5278) RHDV7023R (7023-7043)	RHDV6234R (6234-6253) RHDV5829R (5829-5848) RHDV5682F (5682-5698) RHDV6135F (6135-6154) RHDV6700R (6700-6719) RHDV6654F (6654-6670)

Table 4.1 List of primers used to amplify the capsid gene of RHDV. External primers were used in first round PCR, internal primers were used in second round PCR. The sequencing primers were used in addition to the internal primers to give approximately 500bp of sequence for assembly and analysis

4.3.2 Phylogenetic Analysis

The products produced by the sequence reactions were joined together using Pregap4 and Gap4 (Staden Package), subsequent analysis was carried out using Translate (GCG, Wisconsin Package) and transeq (jEmboss). Sequences not determined in this work, were obtained from the databases and have been described previously (Forrester *et al.*, 2003, Le Gall-Recule *et al.*, 2003, Moss *et al.*, 2002). The VP60 gene sequences were aligned using Pileup (GCG, Wisconsin Package) and ClustalX (Thompson *et al.*, 1997). Phylogenetic analyses were undertaken using PAUP* version 4.0b10 (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 was then used to estimate the Maximum Likelihood tree using iterative heuristic searches with TBR branch swapping algorithms with re-estimation of variable parameters between searches, from the data, where necessary. A neighbor-joining tree was also estimated using the optimal Maximum Likelihood model and settings, and was congruent with the Maximum Likelihood tree. Neighbor-joining bootstrap support (1000 replicates) was calculated for each tree using the Maximum Likelihood settings.

4.4 Results

4.4.1 The evolution of RHDV and the paradox of virulence

Previous phylogenetic analyses (Le Gall *et al.*, 1998, Le Gall-Recule *et al.*, 2003, Moss *et al.*, 2002) of RHDV sequence data demonstrated the evolutionary relationships and global dispersion of these viruses. More sequences are now available, thereby extending the value of the data with which the underlying basis for the epidemiological characteristics of RHDV can be investigated. Figure 4.2 and 4.4 present phylogenetic trees constructed from sequence data within different but overlapping regions of the capsid gene (VP60) of a global collection of RHDV isolates. Until the complete genomes of all these viruses have been sequenced this is the most effective way of comparing all available sequences.

Figure 4.2 presents a phylogenetic analysis of the sequences obtained from RT-PCR products of globally representative samples of rabbit liver, bone marrow or serum. In this tree the sequences relate to nucleotide positions 6165 to 6691 of the capsid gene. Figure 4.2 shows that most of the viruses grouped into relatively closely related clades. However, the viruses within many of these different groups are from widely dispersed geographic regions, presumably illustrating the extent to

which rabbits are being transported for commercial purposes. Strains Ashington, isolated in the UK from a dead rabbit and RCV, isolated in Italy from a healthy rabbit (Fig. 4.2 inset), are widely divergent from all other viruses (85.4% and 85.7% nucleotide identity, respectively) and also from each other (86% nucleotide identity), infering ancient ancestry. They were included to root the viruses in the original phylogenetic analysis (Fig. 4.2 inset) and were then removed for clearer presentation of the tree topology. Comparative sequence alignments of these two viruses with any others showed that Ashington and RCV have a deletion of 6 nucleotides commencing at positions 6222 and 6227 respectively, so that the amino acid sequence "NATN", which is conserved amongst other strains of RHDV, is "- - T D" for Ashington and "N - - N" for RCV. These deletions occur in a region of the capsid protein that is a potential glycosylation site, and would prevent glycosylation in both cases. Whether or not this deletion impacts on virus virulence has not yet been determined but in general differences in glycosylation status between closely related viruses may impact on virus virulence and neutralization characteristics (Chambers, *et al.*, 1998, Shirato, *et al.*, 2004). However, it is important to note that Ashington virus represents a divergent lineage that is ancestral to the Chinese 1984 lineage, and is presumed to have killed the rabbit from which it was isolated. In contrast RCV, the lineage of which is also ancestral to the Chinese 1984 isolate, has been shown by experimental inoculation into domestic rabbits to be attenuated.

In Figure 4.2, the viruses presented in black ink were obtained from rabbits that are presumed to have died as the result of a virulent infection with RHDV. The viruses presented in red ink represent RNA samples of RHDV sequence obtained from healthy rabbits. As can be seen, in each case the sequences obtained from the healthy rabbits are virtually indistinguishable from those assumed to be associated

with virulent infections. Moreover, the RNA sequences from healthy rabbits are widely distributed throughout the tree, in other words they represent viruses that have a worldwide distribution. In addition, many of these sequences were obtained from healthy rabbits that lived between 20 and 30 years before 1984, the time of the first recorded appearance of RHD as a lethal epidemic in rabbits. Thus, prior to the first RHD epidemic in 1984, rabbits distributed throughout the Old World were apparently circulating the RNA of RHDV in a silent or low virulence form.

Importantly, Figure 4.4 shows that the viruses occupying groups 1, 2, 3, and 4, are more divergent than the Chinese lineage from 1984. Since many of these viruses were isolated from rabbits presumed to have died following infection with RHDV, this implies that virulent viruses or earlier lineages of them, may have circulated throughout Europe prior to the Chinese outbreak in 1984. In contrast the viruses in groups 6 and 7 are descendants of the lineage that gave rise to the Chinese 1984 strain, implying that the emergent Chinese strain must have dispersed through these countries causing epidemics despite the possible presence of viruses with more divergent lineages. Therefore, if RHDV was circulating as virulent virus throughout Europe and most of Asia prior to 1984 either it was not recognised or, paradoxically and less likely, these low virulence lineages suddenly "switched" to more virulent forms after emergence of the Chinese strain.

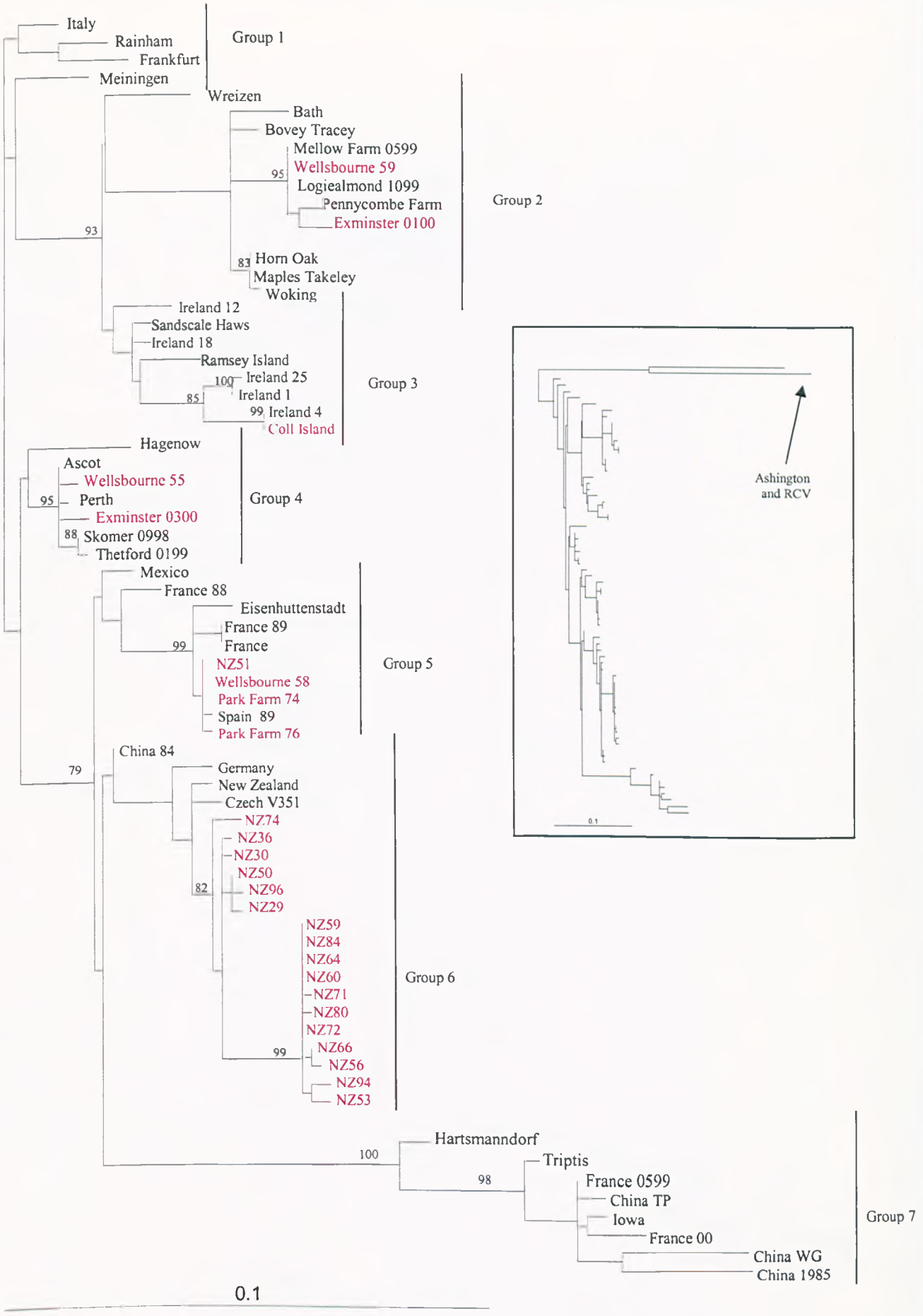

Figure 4.2 (Legend on page 94)

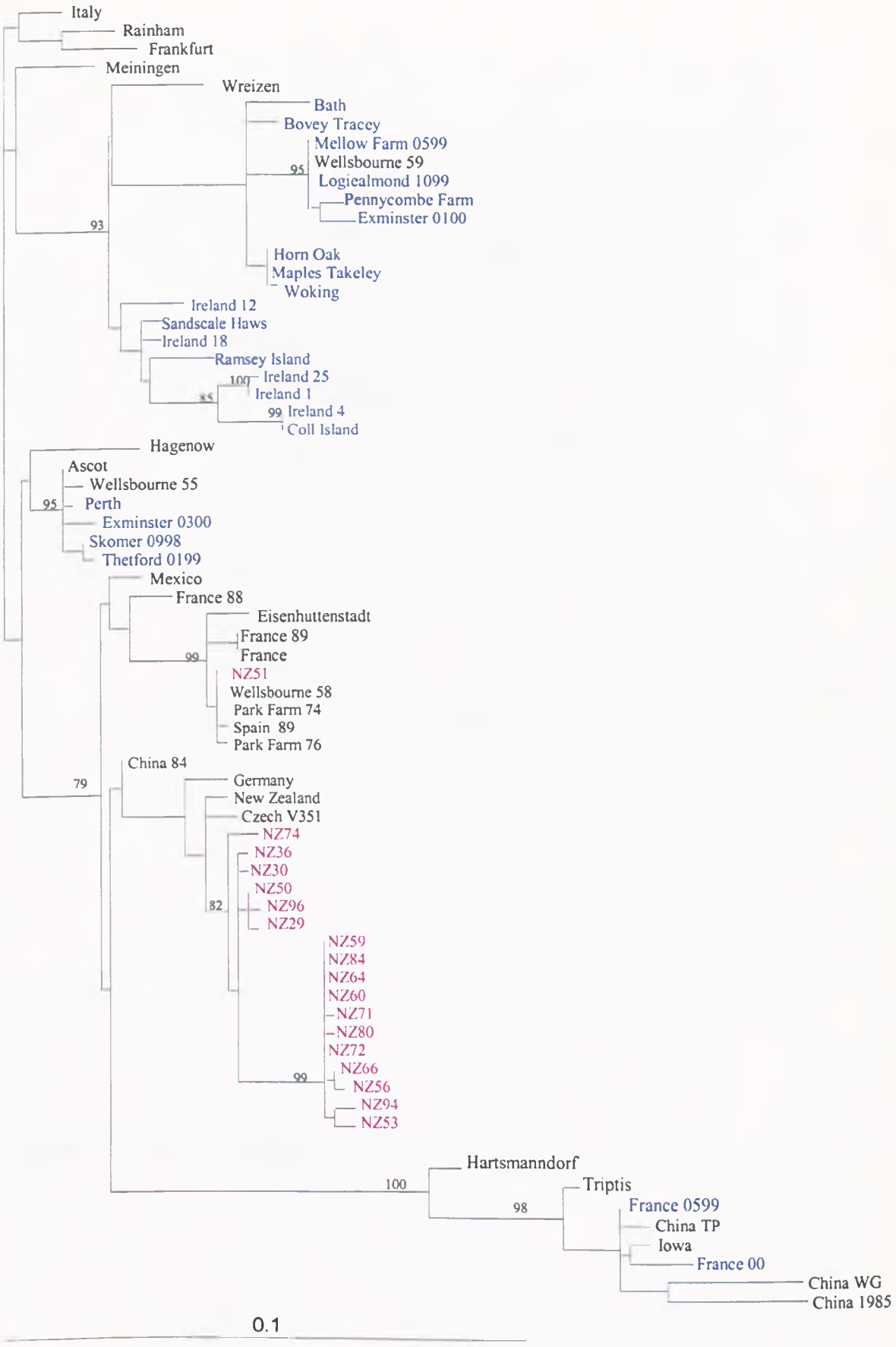

Figure 4.3 (Legend on page 94)

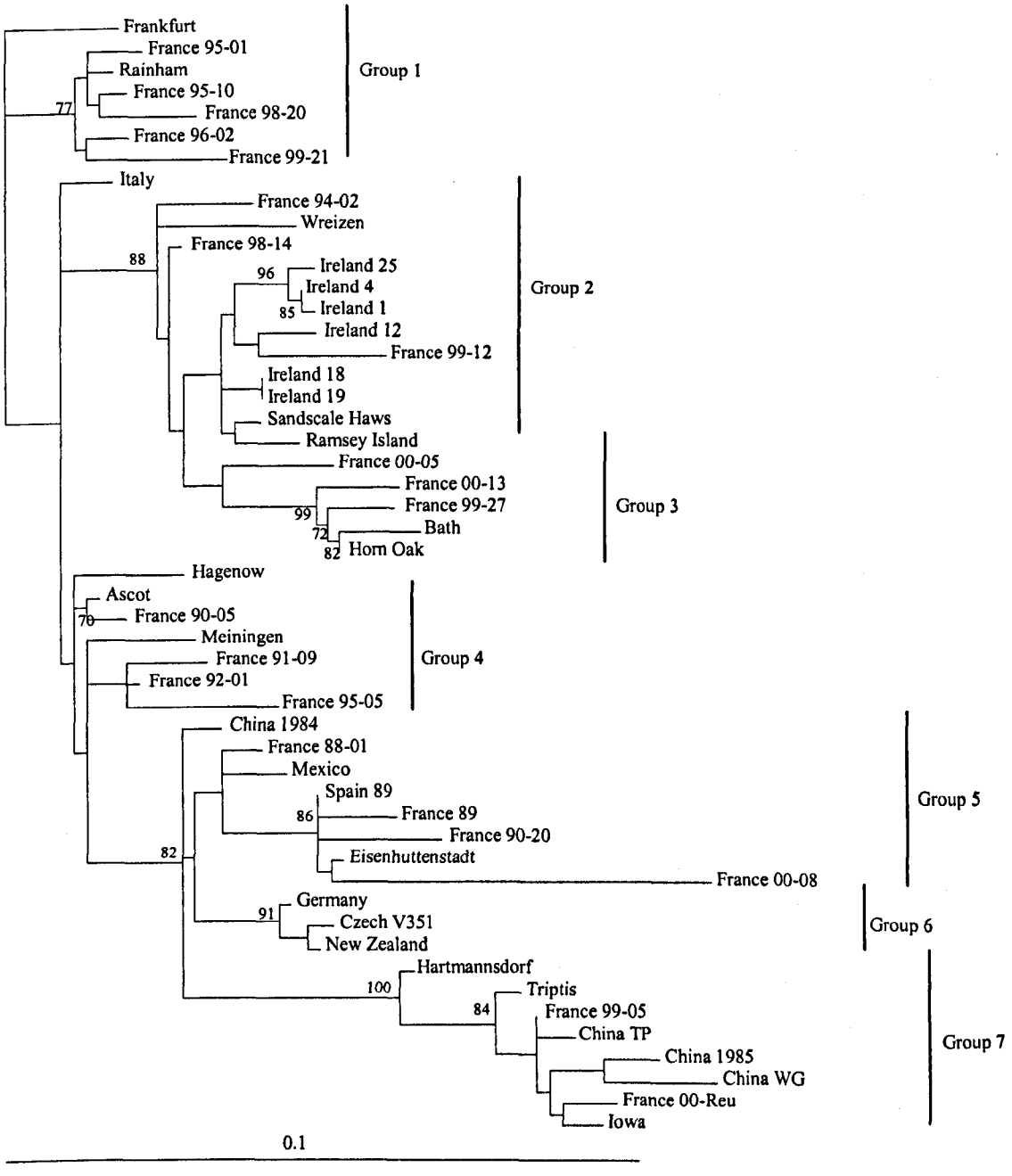

Figure 4.4 (Legend on page 94)

Figure 4.2
Phylogenetic analysis using partial capsid sequence (see Methods) for 71 strains of RHDV. Maximum Likelihood phylogeny was calculated using PAUP* version 4.0b10. The optimal model to use with the data (HKY+I+Γ) was determined using MODELTEST 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 1000 replicates. The tree is highlighted to show viruses isolated from healthy rabbits and those isolated from rabbits presumed to have died from RHDV. Figure 4.2 inset was calculated using 73 strains of RHDV, including Ashington and RCV. Procedure was followed as above and the same model was used. Although the tree topology is slightly different, the changes were not significant under bootstrap analysis. Groups were designed using geographical and historical data and are there as an aid, rather than precise delineations.

Figure 4.3
Showing the known origins of rabbit samples from domestic or wild rabbits for Fig. 4.2. Viruses in blue are known wild rabbit isolates, viruses highlighted in pink are viruses isolated from the wild, but known to be introduced domestic rabbits strains, viruses in black are domestic rabbits or unknown origin.

Figure 4.4
Phylogenetic analysis using partial capsid sequence (see Methods) for 56 strains of RHDV. Maximum Likelihood phylogeny was calculated using PAUP* version 4.0b10. The optimal model to use with the data (TVM+I+Γ) was determined using MODELTEST 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 1000 replicates.

4.4.2 Global Distribution of RHDV

The global distribution of RHDV is largely dependent on the commercial transportation of rabbits and associated materials to different countries as a source of meat and fur. Interestingly, different strains isolated from domestic rabbits from a single country are found in most groups within the tree, for example, Figure 4.4 shows that viruses isolated in France occur in groups 1, 2, 3, 4, and 5, and those isolated in Germany occur in groups 1, 2, 4, 5, 6 and 7. A recent study on French isolates identified several different strains of RHDV by the date of collection rather than their geographic location (Le Gall *et al.*, 1998, Le Gall-Recule *et al.*, 2003). This suggests that there have been several introductions of the virus into France, possibly from Germany, and subsequent spread throughout the population. In contrast, the isolates from wild rabbits in the UK do not show this distribution pattern. All the UK samples from wild rabbits fall into three groups (Groups 2, 3, and 4) irrespective of the date of isolation.

4.4.3 The Irish strains of RHDV form a single group

Figures 4.2 and 4.4 show that the Irish samples from 2000 occupy a monophyletic group (group 2), and these viruses cluster with Irish isolates from 1995 (Collery *et al.*, 1995, Nowotny *et al.*, 1997)(unpublished observation). Thus, subject to the reservation that only a limited number of samples have been analysed, there has been only one introduction of RHDV into Ireland. Moreover, these viruses are most closely related to three British strains, isolated on Ramsey Island, Sandscale Haws (west coast of England) and Coll Island (West coast of Scotland). Also included in this clade are a German isolate (Wreizen) and a French isolate. The branching pattern of the tree (Fig. 4.2) suggests that the virus was introduced into

Ireland from France or Germany and subsequently into England from Ireland and separately from Ireland onto the island of Coll.

It was previously demonstrated that three other English isolates Frensham (AF454033, AF454034), Leominster (AF454031) and Prudhoe (AF454032) are also included in group 2 (Moss *et al.*, 2002). These viruses were isolated at inland sites in the South (Frensham and Leominster) and the North of England (Prudhoe). This dispersal pattern of the Irish strains in England is consistent with the wide and apparently random distribution of different strains of virus as demonstrated by Moss, *et al.*, (2002). Although the origin of the Irish strains is unknown, antibodies from healthy rabbits have been identified from samples taken prior to the epidemic in 1995 (Chasey *et al.*, 1997a, Trout *et al.*, 1997b). This gives added weight to the possibility that the Irish strains have been circulating in Ireland for a considerable period of time in order to generate the branching pattern seen in Figure 4.2.

4.4.4 Wild versus domestic rabbits

Figure 4.3 identifies isolates of RHDV that were isolated from either wild (blue ink) or domestic rabbits (black ink). The viruses shown in pink ink were isolated from wild rabbits but were originally known to be present in domestic rabbits. The tree shows that the viruses from wild and domestic rabbits are closely related and both types are widely dispersed throughout the tree. Figure 4.3 can be divided into two parts, groups 2, 3 and 4 which show the presence of the virus in wild rabbits and groups 5, 6 and 7 which contains viruses isolated mostly from domestic rabbits. In fact even those that are from wild rabbits are descendants of more divergent viruses from domestic rabbits.

4.4.5 RHDV as a bio-control agent

In 1995, the Czech strain of RHDV escaped from Wardang Island onto mainland Australia causing a major epidemic outbreak. Local farmers then deliberately dispersed the virus across their farms in food packages. Subsequently the virus was illegally transported to, and released by, farmers in New Zealand (NZ) (Motha & Clark, 1998). The NZ government quickly legalised this procedure and brought in strict control measures for the release of RHDV. Figure 4.2 (group 6) shows that the Czech strain of RHDV that was deliberately dispersed by farmers in New Zealand, diverged into two closely related but distinct lineages. It was proposed (Forrester *et al.*, 2003) that the more divergent of these two lineages emerged before the NZ government legalised the deliberate use of RHDV as a bio-control agent. It is important to note that the two lineages represent RNA samples collected from the livers of apparently healthy rabbits. Moreover, two of the RNA samples were analysed in more depth and genomic-length sequences were obtained, strongly suggesting that the virus had established long-term infections in these rabbits that did not result in disease (Forrester *et al.*, 2003).

4.5 Discussion

From our interpretation of the RHDV phylogenetic analysis, it appears quite likely that a wide variety of RHDV strains circulated, possibly silently, throughout Europe and Asia amongst rabbits prior to 1984 when the disease first appeared in an epidemic form in China. There are no records of local disease outbreaks due to RHDV in the UK or Europe prior to 1984. However, it is known that rabbit populations are periodically subject to major crashes (Middleton, 1934, Rogers *et al.*, 1994), and there is convincing evidence of antibodies to RHDV in rabbits dating as

far back as 1955 (Chasey *et al.*, 1997a, Moss *et al.*, 2002, Nagesha *et al.*, 2000, Nowotny *et al.*, 1997, O'Keefe *et al.*, 1999, Robinson *et al.*, 2002a, Rodak *et al.*, 1990). These antibodies might be expected to reduce mortality rates following exposure to RHDV. One possible explanation for the failure of observers to recognise RHD in Europe or Asia from the 1950's onwards might have been because RHD was masked by the disease severity and the long-lasting symptoms induced in rabbits by Myxoma virus. Additionally, myxomatosis caused a major reduction in rabbit populations and recovery to pre-myxomatosis levels took until the end of the 1970s. Possibly, the numbers of susceptible rabbits were insufficient to support major epidemics due to RHDV. Some rabbits in Spain and France have antibody to both MYXV and RHDV (Calvete *et al.*, 2002, Marchandeau *et al.*, 2004) supporting the hypothesis that the two viruses may have co-circulated in the UK and Europe prior to 1984.

The sudden appearance of an apparently highly virulent strain of RHDV in 1984 in China was possibly due to the introduction into China of RHDV-infected Angora rabbits imported from Germany. If the virus was introduced in this manner, the imported rabbits were presumably healthy and therefore must have carried the virus silently as appears to have been happening in domestic rabbits for many years prior to 1984. The major epidemic in China that occurred in 1984 could reflect the immunological naivety of the Chinese domestic rabbit population which may not have been exposed to European rabbits carrying RHDV for many years. Similar, high intensity outbreaks were subsequently recorded in domestic rabbits in Spain (Simon *et al.*, 1995, Simon *et al.*, 1998, Villafuerte *et al.*, 1994), Saudi Arabia (Abu Elzein & al-Afaleq, 1999) and up to 40 other countries worldwide (Heneidi Zeckua *et al.*, 1997). In each case it is highly likely that the rabbits involved in these

outbreaks had no or very low RHDV-specific antibodies as they had been reared indoors with little risk of exposure either to wild rabbits or to other domestic rabbits. Nevertheless, following the outbreak in China, and the identification of the virus, other small or large outbreaks in both wild and domestic rabbits in Europe are more likely to have been reported and/or diagnosed. Quite naturally these outbreaks would be interpreted as being due to the rapidly spreading Chinese virus that undoubtedly did spread out of China after its emergence, as can be seen in the phylogenetic trees.

It is now known that RHDV does not always cause fatal infections (Forrester *et al.*, 2003, Moss *et al.*, 2002, Zheng *et al.*, 2002) and genomic-length viral RNA can be carried in the liver of healthy rabbits presumably in the form of a persistent type of infection (Forrester *et al.*, 2003). In addition to immunological naivety, other factors may have influenced the severity of the outbreaks due to RHDV. It has been shown that the effectiveness of RHDV as a bio-control agent depends to a large extent on the environmental conditions under which the virus is released (Barlow *et al.*, 2002, Cooke & Fenner, 2002, Edwards *et al.*, 2002b, Henzell *et al.*, 2002, Mutze *et al.*, 2002), although as yet this is not fully understood. Firstly, in relatively wet areas the virus often causes sporadic or weak epidemics that affect only local rabbit populations. These epidemics may vary in intensity, occasionally leading to local extinction (Barlow *et al.*, 2002). Secondly in drier regions of the world the virus often causes significant reduction of rabbit populations over wide geographic areas (Edwards *et al.*, 2002a, Mutze *et al.*, 2002, Simon *et al.*, 1995, Simon *et al.*, 1998). A recent and surprising observation in Spain is that <40% of wild rabbits now have antibodies to RHDV (Simon *et al.*, 1998). In contrast, >70% of numerous wild rabbit populations in the UK, which has a wetter climate, had antibodies to RHDV (Trout *et al.*, 1997b, White *et al.*, 2004). This reduced level of immunity in Spain

presumably reflects the introduction of young non-immune and highly susceptible rabbits into regions where RHDV is already circulating, but it could also, at least in part, result from stress exerting an immunosuppressive effect on the rabbits in drier climates where food and water are less easily found.

This dichotomy in the effect that RHDV has on rabbit populations suggests that the virus can survive long-term. However, there is no explanation of how the virus persists long-term in the environment. There are several possible ways in which this might occur. Firstly the virus may persist in the environment, in carcasses, faeces and burrows (McColl, *et al.*, 2002b, Shien, *et al.*, 2000). Secondly, we have shown that complete RHDV RNA genomes can be recovered from healthy rabbits (Forrester *et al.*, 2003). Whether or not this represents RNA derived from cells in which virus turnover is very slow, as in persistent infections, remains to be determined. Thirdly, there is now quite convincing evidence that positive-strand RNA viruses can utilise reverse transcriptase to produce the equivalent of episomal DNA (Crochu *et al.*, 2004). If such a mechanism does exist for caliciviruses this would indeed provide a stable form of nucleic acid that could be perpetuated for very long periods of time. By analogy with herpes latency, reactivation of the virus could occur under conditions that induce stress in the rabbit. This is not unprecedented, as cases of sudden death in rabbits held in clean laboratory environments have been observed. Post-mortems of these rabbits revealed symptoms not unlike those relating to RHD (E.Gould – unpublished observation). A fourth, but more speculative mechanism for long-term virus survival utilises the concept of homeostatic regulation of virus replication through the production of quasispecies (Sallie, 2005).

Finally, RHDV initially appeared to be suitable for use as a bio-control agent, i.e. to reduce rabbit populations in regions where their numbers were excessively

high. However, within a relatively short period of time after the deliberate release of the virus into what was thought to be virgin territory, its effectiveness has already begun to decline. Our results suggest that the deliberate release of the virus as a bio-control agent will be more effective in those areas where climate and population size combine to give the greatest impact. Following its release it is likely that RHDV will initially reduce the rabbit population and will then persist in the reduced population with low level disease incidence. Subsequently, when the proportion of susceptible rabbits reaches a critical level, new epidemic outbreaks are likely. We propose that climate, time between epidemics and geographic location will all influence the success of the bio-control measures. It is likely that controlled release of the virus in areas not having experienced an epidemic outbreak for a relatively large number of years will increase the effectiveness of the bio-control programme.

Chapter 5

Phylogenetic analysis of Saudi Arabian and Bahrainian strains of Rabbit haemorrhagic disease virus: did RHDV emerge simultaneously in Europe and Asia?

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5.1 Summary

Following the emergence of RHDV in China in 1984 it was assumed that RHDV dispersed across the world causing numerous epidemics. Two separate outbreaks occurred in domestic rabbits in Saudi Arabia and Bahrain. Whilst the Saudi Arabian epidemic was thought to be attributable to the importation of infected material the origin of the Bahrain epidemic was more ambiguous. The origin of these outbreaks was determined by phylogenetic analysis. The results show that the Saudi Arabian outbreak is a descendant of the 1984 Chinese strain of RHDV, whereas the Bahrain strain is a direct descendant of a European strain of RHDV and therefore is distinct from the Chinese 1984 strain. This implies that RHDV emerged at least twice in the last twenty years, once in China, and from the phylogenies, probably more than once in Europe. This may partly explain the wide level of divergence observed between different strains of RHDV.

5.2 Introduction

The first major epidemic of Rabbit haemorrhagic disease was observed in China in 1984 (Liu *et al.*, 1984). It swept through the Chinese domestic rabbit population killing 14 million animals in nine months (Xu, 1991). The disease was characterised by acute fulminating hepatitis with death usually occurring between 36 – 72 hours post-infection. The aetiological agent was classified as a calicivirus (Ohlinger *et al.*, 1990, Ohlinger & Thiel, 1991, Parra & Prieto, 1990).

Following the outbreak in domestic rabbits in China, rabbit haemorrhagic disease virus (RHDV) appeared to disperse westwards across Europe (Marcato *et al.*, 1988), Africa (Kpodekon & Alogninouwa, 1998), and the middle east (Abu Elzein & al-Afaleq, 1999), and southwards across Asia (Mizoguchi *et al.*, 2003, Park *et al.*, 1987). By the late 1990's it had been reported in domestic rabbits in 40 countries (Heneidi Zeckua *et al.*, 1997). However, the virus was not observed to cause disease in wild rabbits before 1988 (Cooke, 2002, Villafuerte *et al.*, 1994). Subsequently, it appeared to disperse throughout the wild rabbits populations of Europe (Chasey *et al.*, 1997a, Marchandeau *et al.*, 1998).

Several highly lethal outbreaks have occurred in domestic rabbitries in countries where there are no or few wild rabbits (Berninger & House, 1995, Gregg *et al.*, 1991). Recently, two outbreaks occurred in the Middle East, in Saudi Arabia (Abu Elzein & al-Afaleq, 1999) and separately in Bahrain (Dr. Abubakr – personal communication). The outbreak in Saudi Arabia killed 100% of rabbits in a rabbitry within 10 days. It occurred in November 1996, and was followed for a further three months with sporadic outbreaks in other rabbitries across the country with mortality rates frequently reaching 100%. It lasted for three months (Abu Elzein & al-Afaleq, 1999). An apparently similar outbreak occurred in the Kingdom of Bahrain in May

2001, at a private farm where wild rabbits are known to inhabit the surrounding areas. The outbreak lasted for two weeks and caused a high mortality rate. In this case there were no subsequent outbreaks in other rabbitries probably because of the relative isolation of the rabbitry in which the epidemic occurred. The relatively long period of 4 years between these two outbreaks suggested to us that they may not necessarily have been caused by the same virus.

Previous work has demonstrated that there are many strains of RHDV circulating in different rabbit populations, most of which show distinct epidemiological, pathogenetic and genetic characteristics (Forrester *et al.*, 2003, Le Gall *et al.*, 1998, Le Gall-Recule *et al.*, 2003, Moss *et al.*, 2002, Nowotny *et al.*, 1997). Nevertheless recent molecular epidemiological studies have shown that the majority of these viruses can be grouped into at least two phylogenetic lineages, one represented by the Chinese 1984 strain and its descendant lineages whilst the other is represented almost exclusively by European viruses. These groupings highlight an apparent paradox, i.e. the European isolates of RHDV do not appear to be descendants of the lineage that produced the epidemic in China in 1984 (Chapter 4). The purpose of this study is therefore twofold, firstly to identify the genetic origins of the Saudi Arabian and Bahrainian viruses and secondly, to attempt to explain the link between the European and Asian lineage viruses. It is hope that this analysis will extend our understanding of the epidemiology, evolution and dispersal of RHDV.

5.3 Materials and Methods

Post-mortem examination of rabbits that died in Saudi Arabia and Bahrain were carried out immediately following their death. Livers, lungs and kidneys were aseptically collected in sterile containers virological laboratory examination. Duplicate samples were also collected in 10% formol saline for histopathological examination. Samples for virological examination were preserved in virus storage medium and frozen at -70°C. For molecular analysis, RNA was extracted from 100μg of liver tissue using the RNAgents kit (Promega) following the recommended protocols. The primer sequences for RT-PCR were designed from published RHDV data (Boga *et al.*, 1992, Gould *et al.*, 1997, Meyers *et al.*, 2000, Rasschaert *et al.*, 1995) and are described in Table 3.2 (p66). First strand cDNA synthesis was performed using Superscript II reverse transcriptase (Life Technologies) and the reverse external primer. The DNA was amplified by nested PCR, utilising the external and internal primers described in Table 3.2 (p65) yielding a product of between 1000 and 1750bp. The resulting product was gel purified and both strands were sequenced using a PE Biosystems cycle sequencing kit with the sequencing primers producing approximately 500bp of sequence for analysis.

The sequences were assembled using Pregap4 and Gap4 (Staden Package), subsequent analysis was carried out using Translate (GCG, Wisconsin Package) and transeq (jEmboss). Sequences not determined in this work were obtained from the database and have been included in previous analyses (Forrester *et al.*, 2003, Le Gall-Recule *et al.*, 2003, Moss *et al.*, 2002). The VP60 gene sequences were aligned using Pileup (GCG, Wisconsin package) and ClustalX (Thompson *et al.*, 1997). Phylogenetic analyses were undertaken using PAUP* version 4.0b10 (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 was then used to estimate the Maximum Likelihood tree using iterative heuristic searches with TBR branch swapping algorithms with re-estimation of variable parameters between searches, from the data, where necessary. A neighbour-joining tree was also estimated using the optimal Maximum Likelihood model and settings, and was congruent with the Maximum Likelihood tree. Neighbor-joining bootstrap support (1000 replicates) was calculated for each tree using the Maximum Likelihood settings.

5.4 Results and Discussion

Full-length genomic sequences of the viruses isolated in Saudi Arabia and Bahrain were developed using RT-PCR sequencing utilising the primers described in Table 3.2 (p66). The resulting sequences were aligned with another 71 RHDV sequences obtained from GenBank using ClustalX (Thompson *et al.*, 1997) and the alignment was finally adjusted manually. In order to obtain the maximum phylogenetic information three trees were constructed using PAUP*, one corresponding to nucleotides 6165-6691, one based on the entire capsid protein, and one that utilised the entire genomic sequence. Only the tree based on partial capsid sequence is presented (Fig. 5.1) as this provides the largest number of viruses for comparison. However, the trees based on complete capsid or complete genomic sequence produced the same topology. Previous analyses have included the strains Ashington (AF454050) and RCV (X96868) (Capucci *et al.*, 1998), which are significantly different from the other strains (Forrester *et al.*, 2003, Moss *et al.*, 2002). In this analysis, the tree was firstly constructed with these viruses included, they were then removed and the tree was re-constructed, producing the same

topology but more robust bootstrapping. The results of the analysis are presented in Figure 5.1. For clarity of presentation the viruses are divided into groups 1 to 3. As can be seen group 1 contains only European viruses, the most divergent of which were isolated in Germany (Frankfurt) and England (Rainham).

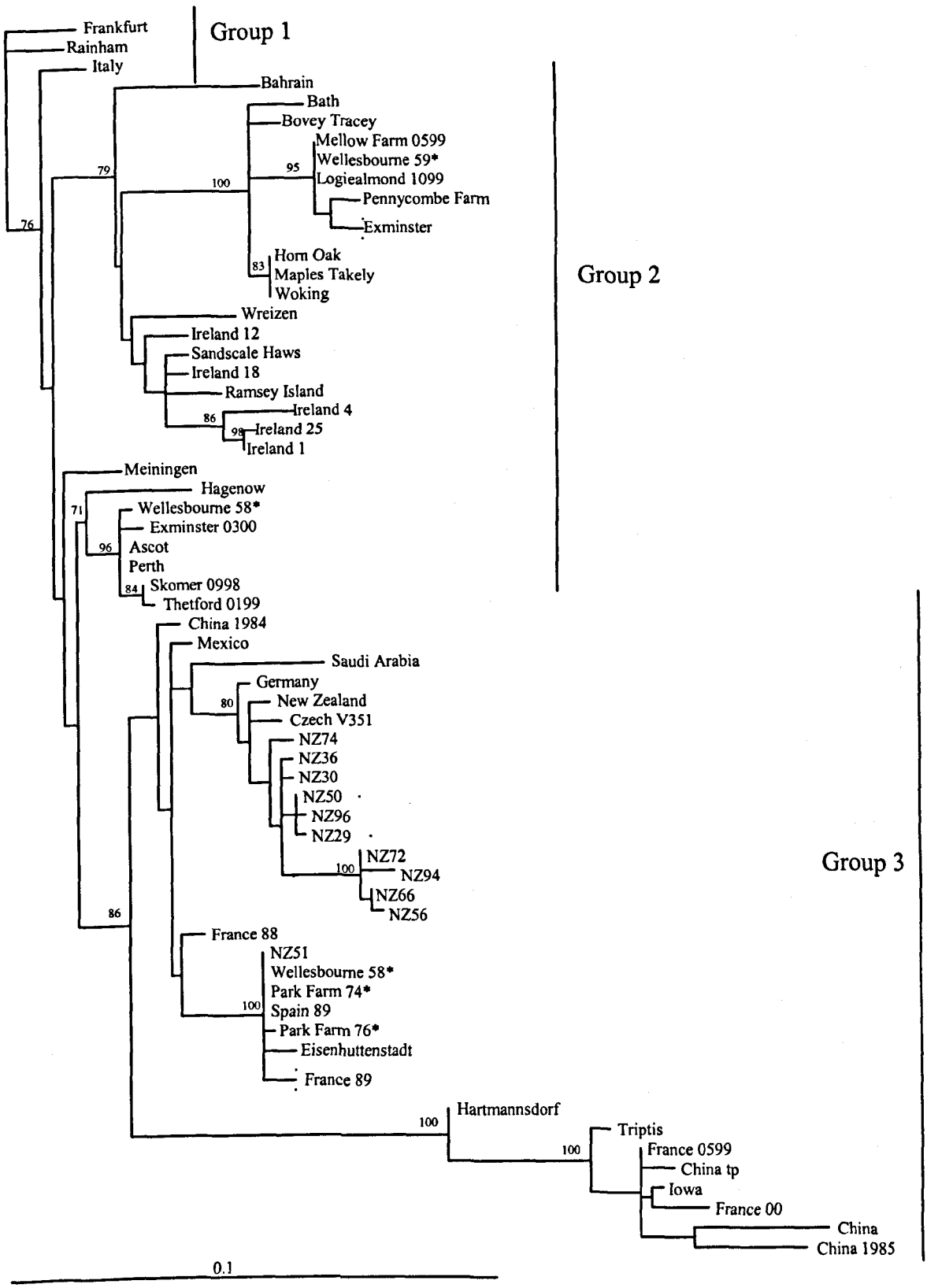

Figure 5.1
Phylogenetic analysis using partial capsid sequence (see Methods) for 31 strains of RHDV. Maximum Likelihood phylogeny was calculated using PAUP* version 4.0b10. The optimal model to use with the data (TVM+Γ) was determined using MODELTEST 3.06, and the variable parameters were estimated from 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 1000 replicates.

The tree does not resolve these two viruses illustrating that they are very closely related. However, the third isolate in this group, i.e. from Italy is significantly less divergent implying separation from the German and English strains for a relatively long period of time.

With the exception of the Bahrain isolate, group 2 contains only European viruses. It is important to note that the Bahrain isolate has evolved distinctly from the European isolates, implying that although they have common ancestry, the divergence point is not a recent event. Group 2 also consists of two major sub-groups, those rooted by the Bahrain isolate and those rooted by the Meiningen isolate.

The group 3 viruses branch on the most recent lineage of those analysed. The Chinese isolate that was responsible for the first reported outbreak in 1984 is included in group 3, in other words the lineage emerged more recently than the lineages of any of the viruses in groups 1 or 2. The tree shows that the Saudi Arabian isolate occupies a descendant lineage of the Chinese 1984 strain. The tree also shows that whilst the Saudi Arabian isolate is related to the Czech isolate that was used in the bio-control studies in Australia and New Zealand (Asgari *et al.*, 1999, Gould *et al.*, 1997, Motha & Clark, 1998), it separated before the Czech V351 virus emerged. Many of the other viruses in group 3 are European isolates, although this group also contains isolates from Mexico, Iowa and China (China tp and China 1985) all of which were presumably introduced into these countries through the importation of domestic rabbits or contaminated rabbit foodstuff.

The tree also shows that some UK viral lineages (Park Farm and Wellesbourne) are directly descended from the lineage corresponding to the group 3 China 1984 isolate. Since the Park Farm and Wellesbourne sequences were isolated from serum stored in the 1950's and 1970's, this means that the China 1984 isolate was circulating, apparently harmlessly in the UK and possibly also in Europe many years before it caused the outbreak in China. However, although many different strains of RHDV have been identified in the UK, this Chinese epidemic virus has never been identified as a virulent infection in the UK.

The long branch lengths of the viruses in group 3 that are rooted by the strain Hartmannsdorf could reflect recombination between strains of RHDV since we have identified recombination in Hartmannsdorf and other strains of RHDV (Chapter 7). Regardless of the cause of this long branch length, the other isolates in this sub-group (Triptis, France, China tp, China 1985 etc.) diverged independently of the epidemic Chinese 1984 strain. As in the case of the group 1 and group 2 viruses this infers that epidemic strains of RHDV have emerged more than once.

Finally it is important to note that viruses identified with a * represent previously published sequences of RNA obtained as archived sera from healthy rabbits that lived before 1984 (Moss *et al.*, 2002). These RNA sequences are found in groups 2 and 3 demonstrating that both virulent and avirulent RHDV lineages circulated before 1984 even though the disease was not recognized.

Prior to 1984, myxomatosis was the only recognised viral disease of rabbits. When RHDV unexpectedly appeared killing millions of rabbits (Liu *et al.*, 1984) it was assumed to have emerged in China and all subsequent epidemics were understandably attributed to this virus radiating southwards and westwards across Asia and Europe. However, it is now known that prior to 1984, healthy rabbits in

many regions of Europe had RHDV- specific antibodies (Chasey *et al.*, 1997a, Moss *et al.*, 2002, Nagesha *et al.*, 2000, O'Keefe *et al.*, 1999, Rodak *et al.*, 1990) and in some cases RHDV-specific RNA was also detectable in the serum of these archival collections (Moss *et al.*, 2002). Moreover, although the virulent Czech strain of RHDV was deliberately released as a bio-control agent in New Zealand, only a few years later approximately 50% of sampled healthy rabbits contained genomic-length RHDV-specific RNA in their livers (Forrester *et al.*, 2003, Zheng *et al.*, 2002), but infectious virus did not appear to be causing disease in the region at the time of collection of these rabbits. Therefore the original assumption that RHDV emerged as a uniquely virulent virus in China in 1984 and then radiated across Asia and Europe is too simplistic.

The phylogenetic analysis presented in this paper shows that the Bahrain isolate of RHDV is a direct descendant of the group 2 European lineage which contains many isolates that have been associated with epidemics in Europe and many of these lineages pre-date 1984. On the other hand, the Saudi Arabian isolate is descended from the more recently diverged group 3 lineage that includes the Chinese isolate from 1984. This means that epidemic RHDV must have emerged at least twice in the past, i.e. at least once in Europe and at least once in China. However, the European virus emerged before the Chinese virus even if it did not cause disease at the time of its emergence. Since there are no reports of disease corresponding to that typified by rabbit haemorrhagic disease, prior to the 1984 Chinese outbreak, it appears that different lineages of RHDV started to produce epidemics in Europe and Asia within a similar time period probably within the early 1980's.

How can we explain this? One possibility is that the disease first appeared in China in 1984 because a group 2 European virus was introduced, via healthy

imported Angora rabbits (Xu, 1991), into the farmed rabbits in China, which were immunologically totally naïve to RHDV and therefore highly susceptible to the introduced virus. This virus then radiated westwards and southwards, causing major outbreaks in non-immune domestic rabbit populations, typified by the epidemics in Spain (Villafuerte *et al.*, 1994) and Saudi Arabia (Abu Elzein & al-Afaleq, 1999). On the other hand this virus caused only local spasmodic outbreaks in wild rabbit populations where a significant proportion were immune following exposure to group 2 European RHDV strains that were circulating concurrently. Disease was first seen in domestic rabbits in the UK in 1992 (Fuller *et al.*, 1993) but the aetiological agent was derived from the group 2 lineage. Thus the virus that caused spasmodic disease outbreaks and gradually dispersed throughout the UK in the early 1990's was derived from European lineage viruses, not the Chinese 1984 epidemic lineage, even though it was present in the UK (Moss *et al.*, 2002).

In summary, taking into account the group 1 virus clade and also Ashington virus which is genetically distinct from the other RHDV lineages (Moss *et al.*, 2002), RHDV has probably emerged several times as a virulent virus that causes lethal RHD in rabbits. In each case, the emergent virulent virus appears to have caused epidemics in regions where immunity to RHDV was already present in a significant proportion of the animals. This is not unprecedented since there is evidence that immune animals can be fatally infected by RHDV (Calvete *et al.*, 2002, Marchandeau *et al.*, 2005). On the other hand, the domestic rabbits that are sold commercially are now tested for antibodies to RHDV and only negative animals are supplied to customers. These are the rabbit populations in which very high fatality rates are observed as for example in Saudi Arabia and Bahrain. A high proportion of wild rabbits in many parts of Europe will have had previous exposure to the virus

and this will explain why epidemics remain relatively localised and do not eradicate entire populations over large land masses. Using the same arguments and bearing in mind that prior to 1984, most rabbits in the UK and in Europe would have been exposed to RHDV even if disease was not recorded, it is not surprising that major epidemics involving high fatality rates were not observed in many countries.

We now need to identify the factors that led to the repeated emergence of virulent RHDV from apparently harmless virus that circulates amongst most rabbit populations.

Chapter 6

Evidence of long-term persistence of Rabbit haemorrhagic disease virus in healthy rabbit on Lambay Island, Eire and detection of recombination amongst disparate strains of virus

Manuscript in preparation for submission to Journal of General Virology

6.1 Summary

With two notable exceptions (Ashington, and RCV), strains of RHDV from around the world share significant homology. Here we report by phylogenetic analysis, the identification of another disparate strain, isolated from healthy rabbits on Lambay Island off the east coast of Eire. ELISA tests showed high titre RHDV-specific antibodies in >85% of rabbit sera, confirming that the virus circulates among rabbits on Lambay Island. A recombinant strain of RHDV was also identified. It appears to have arisen through recombination between two genetically different strains of RHDV, implying the existence of an additional divergent strain of RHDV. The presence of the Lambay strain in an isolated wild rabbit population provides the most convincing evidence that RHDV may survive long-term by producing persistent infections.

6.2 Introduction

The sudden emergence of RHDV as an epidemic disease in China in 1984 (Liu *et al.*, 1984) led to the assumption that it was a new, highly virulent disease of the European rabbit. Initially the virus was presumed to have been introduced into China via a shipment of German Angora rabbits (Cooke, 2002, Xu, 1991). Whilst we have recently reported that the evolutionary origin of the Chinese lineage appears to be European (Chapter 5), no virus in Germany has yet been identified as a direct ancestral lineage of the 1984 Chinese epidemic virus. It is also important to note that there were no reports of epidemic RHDV in Germany at the time of the first outbreak in China. This could be interpreted as indicating either that the Chinese virus had been introduced from a source other than Germany, or the virus could have been carried benignly in the European rabbit, i.e. without causing severe disease. It seems most unlikely that the virus originated from animals other than rabbits because detailed research in Australia has demonstrated clearly that no other animal species appears able to support its replication (Lenghaus *et al.*, 1994). Indeed it is generally accepted that RHDV has only one susceptible mammalian host, namely the European rabbit (*Oryctolagus cuniculus*). If we rule out long-term virus survival outside infected hosts, whilst there has never been definitive evidence that contagious RHDV can persist in healthy rabbits it seems reasonable to suggest that the virus may circulate in rabbits in at least two forms. Firstly, the virus may survive in an avirulent form, equivalent either to a persistent infection or to a low level infection without overt symptoms. Alternatively, the virus may circulate in a highly virulent form which typically causes epidemics of varying proportions throughout the year, either resulting in death from acute fulminating hepatitis and general organ failure within 36-72 hours post infection or when infected rabbits have pre-existing

immunity, the rabbit recovers from the infection. For this latter scenario, one would have to assume that during inter-epidemic periods, the virus still causes a small number of deaths that are not recorded by observers.

At one time it appeared that this apparent discrepancy had been resolved with the identification of an apparently avirulent virus that was isolated in an Italian rabbitry in 1996 (Capucci *et al.*, 1996b) and was named Rabbit calicivirus (RCV). This virus was shown experimentally to be significantly less virulent for rabbits than other strains of RHDV, and it was suggested that it might represent the pre-1984 virus (Capucci *et al.*, 1996b). However, to date RCV has not been identified in domestic rabbits anywhere other than Italy. Moreover, all other viral RNA sequences that have been extracted from healthy rabbits have been shown to be genetically very closely similar to extant strains that caused virulent outbreaks (Forrester *et al.*, 2003, Moss *et al.*, 2002, Zheng *et al.*, 2002).

Prior to this report, one other strain of RHDV had been identified that is genetically substantially different from RHDV, namely strain Ashington (AF454050) (Moss *et al.*, 2002). The viral RNA of this virus was extracted from the serum of a dead rabbit. Ashington virus shares approximately 80% homology either with RCV or with other extant strains of RHDV. Although initially these two strains (Ashington and RCV) may have been considered to be atypical, i.e. not representative of the genus as a whole, the phylogenetic analysis suggests that they represent the most divergent lineages of RHDV that emerged many years before the 1950's, the time of the most ancient archival sera containing RHDV-specific RNA (Moss *et al.*, 2002).

The present study describes the discovery of another disparate strain of RHDV from an island off the eastern Irish coast where rabbit haemorrhagic disease had never been observed and the introduction of Myxoma virus on several occasions, as a bio-control agent has failed to control the rabbit population density which is now extremely high. As will be shown the status of the Lambay strain of RHDV as an apparently completely innocuous virus provides strong support for the concept of persistence in RHDV. In addition we report the identification of recombination between two disparate strains of RHDV. The discovery of this recombinant virus implies the existence of at least one additional widely divergent strain of RHDV, making a total of four thus far. The discovery of such "fossils" provides excellent material for further studies on the evolution, epidemiology and pathogenesis of RHDV.

6.3 Materials and Methods

6.3.1 RT-PCR sequencing

Samples of blood were collected from apparently healthy rabbits on Lambay Island (OS 3 320 510). The erythrocytes were removed by centrifugation and RNA was extracted from the sera using the RNAgents kit (Promega) following the manufacturer's' instructions. Primers for RT-PCR were designed from known sequences based on the RHDV capsid protein VP60 (Boga *et al.*, 1999, Capucci *et al.*, 1996b, Rasschaert *et al.*, 1995) and corresponded to nucleotides (nt) 6069-6114 (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. A 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 cDNA product of 573 bp. For the PCR a total of 30 cycles of 90°C for 40s, 55°C for 40s and 72°C for 2 mins was used for both sets of primers. These PCR products were gel-purified and both strands sequenced using the BigDye v3.1 Cycle sequencing kit (Applied Biosystems) following the manufacturer's' instructions, producing DNA fragments of approximately 523bp for analysis.

6.3.2 Phylogenetic Analysis

The sequences were aligned with published representative RHDV sequences (Table 6.1) using ClustalX (Thompson *et al.*, 1997). Phylogenetic analyses were carried out using PAUP* version 4.0,10b (Swofford, 2000). The optimal model for the data was estimated for the dataset using MODELTEST v3.6 (Posada & Crandall, 1998). The optimal model (HKY+Γ) was then used to estimate the Maximum Likelihood tree using iterative heuristic searches with TBR branch swapping algorithms with re-estimation of variable parameters between searches, from the data, where necessary. A neighbor-joining tree was also estimated using the optimal Maximum Likelihood model and settings, and was congruent with the Maximum Likelihood tree. Neighbor-joining bootstrap support (1000 replicates) was calculated for each tree using the Maximum Likelihood settings.

Sequence	Reference	Accession No.
Ashington	Moss *et al.* (2002)	AF454050
Ascot	Moss *et al.* (2002)	AF454039
Bath	Moss *et al.* (2002)	AF454024
Bovey Tracey	Moss *et al.* (2002)	
China 1984	Yan *et al.* (unpublished)	AF402614
Czech V351	Gould *et al.* (1997)	U54983
France 89	Rasschaert *et al.* (1995)	Z29514
Frankfurt	Schirrmeier *et al.* (unpublished)	Z15424
Germany	Meyers *et al.* (1991b)	NC_001543
Hagenow	Schirrmeier *et al.* (unpublished)	Y15441
Hartmannsdorf	Schirrmeier *et al.* (unpublished)	Y15425
Ireland 12	Forrester *et al.* (submitted)	AY926883
Italy	Rossi *et al.* (unpublished)	X87607
Meiningen	Schirrmeier *et al.* (unpublished)	Y15426
Park Farm 74	Moss *et al.* (2002)	AF454047
Park Farm 76	Moss *et al.* (2002)	AF454048
Rainham	Riboli *et al.* (unpublished)	AJ006019
Ramsey Island	Moss *et al.* (2002)	AF454036
RCV	Capucci *et al.* (1996)	X96868
Spain 89	Parra *et al.* (1993)	Z49271
Wellesbourne 55	Moss *et al.* (2002)	AF454040
Wellesbourne 58	Moss *et al.* (2002)	AF454049
Wellesbourne 59	Moss *et al.* (2002)	AF454007
Wriezen	Schirrmeier *et al.* (unpublished)	Y15427

Table 6.1
Representative sequences aligned with Lambay to give an indication of the relationship of Lambay with other strains of RHDV

6.3.3 ELISA Tests

Enzyme-linked immunoassays to detect RHDV-specific and MYXV-specific antibodies in rabbit sera were carried out as described in Chapter 2.

6.3.4 Analysis used to determine recombination

Recombination was identified using the SIMPLOT program version 3.5.1 (Lole *et al.*, 1999, Ray, 1997), with bootscanning analysis (Salminen *et al.*, 1995). During the bootscanning procedure, the alignment was divided into sequential segments of 200 nt, overlapping every previous segment by 180 nt. A bootstapped

analysis using 100 replicates was applied to each segment by using the Neighbour-Joining algorithm, with Maximum Likelihood settings and a transition/transversion ratio as determined by MODELTEST 3.06 (Posada & Crandall, 1998). Sequences were compared against reference sequences and the probability of the sequence falling within a specified group was calculated along the genome. Bootstrap scores of >70% are usually thought to indicate significant support.

6.4 Results

6.4.1 Location of Lambay and history of rabbits on the Island

Lambay Island is situated approximately 4km of the Fingal coast of Eire (Fig. 6.1). Although rabbits were believed to have been present on the Island prior to the introduction of MYXV in the 1950's, the population was decimated by myxomatosis then finally wiped out due to predation. Healthy rabbits were subsequently imported onto the island in approximately 1985 from the Dublin area of Eire, as far as can be determined this has happened only once (Dr. R. Trout – personal communication). Since then the rabbits have progressively increased in numbers reaching "plague" proportions, during the past few years. The impact of the rabbits has recently been so severe that the level of suitable cattle-grazing land has been significantly reduced. Attempts to reduce the rabbit densities were made by introducing infected rabbits known to have myxomatosis, but this had little or no effect on the rabbit numbers. It is important to note that there has been no sign of RHDV since the rabbits were first introduced in 1985 and there are no reports of RHDV in or near to Dublin even though RHDV has been recognised, in other parts of Ireland, since 1995 (Collery *et al.*, 1995).

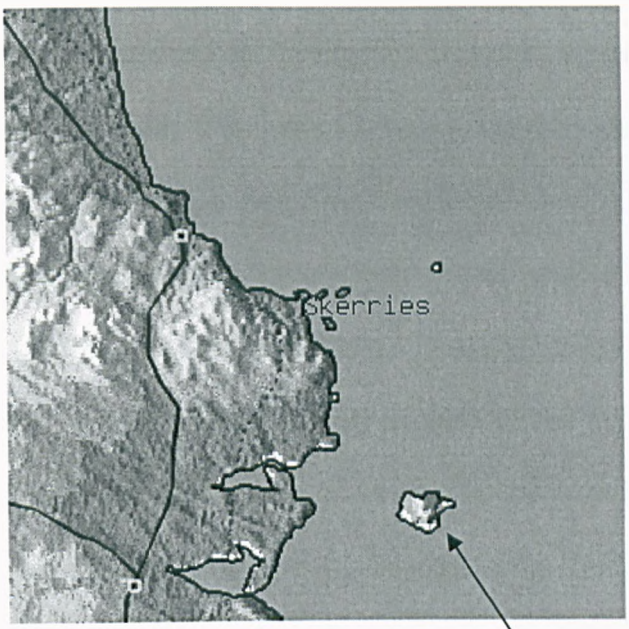

Figure 6.1
Map showing the position of Lambay Island in relation to the eastern coast of Ireland

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6.4.2 RT-PCR and sequencing of extracted RNA and phylogenetic analysis

Sera from 11 rabbits shot on Lambay Island were tested for the presence of RHDV-specific RNA using the RT-PCR procedure described in methods. Two samples produced amplified cDNA of the anticipated size (527bp). One of these samples produced DNA sequence equivalent to RHDV, when compared to other known sequences of RHDV. This sequence consisting of 527bp was aligned with representative RHDV sequences (Table 6.1) and the data were used to construct a phylogenetic tree using PAUP* (Fig. 6.2). In addition to the representative strains of RHDV collected worldwide, the highly divergent strains Ashington and RCV were

also included in the analysis. The tree shows the virus organised for convenience as 5 groups in which groups 2 to 4 comprise European viruses, plus one strain of virus from Bahrain, and group 5 includes Chinese isolates as well as European, American, Middle Eastern, Australian and New Zealand isolates. The Lambay Island virus diverged with Ashington (84.9% nucleotide identity) and these two viruses diverged with RCV (81.0% nucleotide identity). Lambay virus shared 82.8% identity with the Frankfurt isolate which is closely similar to the level of divergence between Ashington, RCV and the group 2 viruses. The tree topology implies that the Ashington and Lambay Island strains emerged after divergence of the lineage from RCV, possibly separating when the virus was introduced either into Ireland or England.

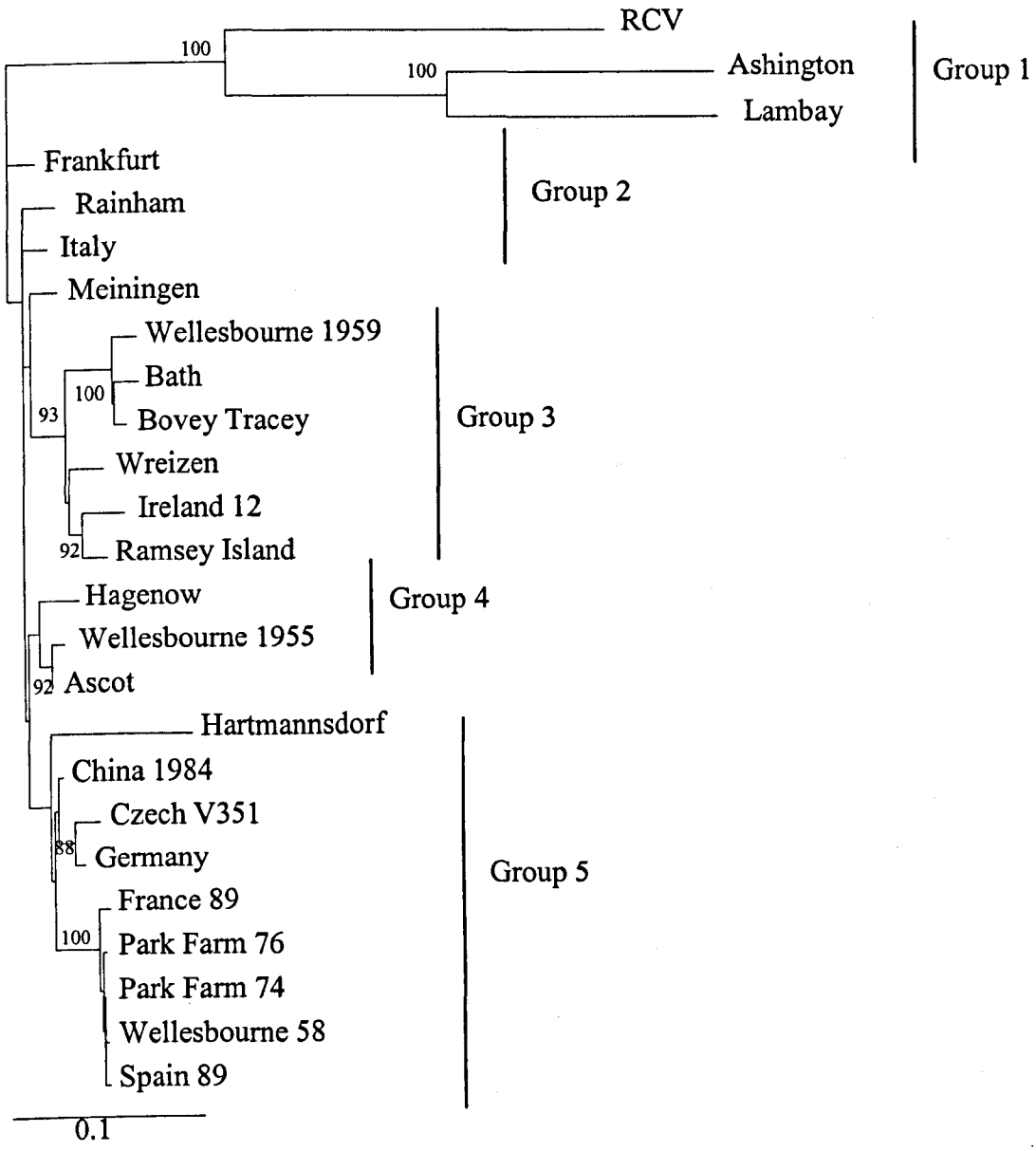


Figure 6.2
Phylogenetic analysis using partial capsid sequence (see Methods) for 25 strains of RHDV. Neighbour-Joining phylogeny was calculated using PAUP* version 4.0b10. The optimal model to use with the data (HKY+Γ) was determined using MODELTEST 3.06, and the variable parameters were estimated from 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 1000 replicates. Both Maximum Likelihood and Neighbour-Joining trees were congruent.

It is important to note that the Lambay Island strain is a significantly different virus from the other viruses isolated in Ireland (Chapter 4), which were first described in 1995 (Nowotny *et al.*, 1997) and have now been studied phylogenetically in more detail (Chapters 4 & 5). The tree shows that these Irish viruses are closely related to other European strains and emerged more recently than the Lambay Island lineage. Several specific observations have arisen from these data. Firstly, the wide divergence of the Lambay Island lineage suggests that it emerged many years before the extant RHDV epidemic strains that circulate globally. Moreover, the fact that the RNA extracted from the Lambay sample was identified in an isolated rabbit population that has never been observed to show symptoms of RHDV suggests that this virus has circulated harmlessly amongst rabbits in Eire for a considerable period of time, and almost certainly prior to the introduction of the other Irish viruses which have been discussed above (Collery *et al.*, 1995)(Chapter 4). Thirdly, the serological evidence of RHDV-specific antibodies in healthy rabbits supports the conclusion that a virus with unique sequence circulates harmlessly, either as an attenuated contagious virus or as a persistent infection amongst rabbits on Lambay Island. Fourthly, rabbits that appear to be insusceptible to the Lambay Island strain of RHDV also appear to be resistant to MYXV. Whether or not this resistance to two known highly pathogenic viruses is significant awaits further investigation. Finally, as yet it is unclear if this virus could ever cause an epidemic either in the Lambay Island rabbits, in rabbits on the Irish mainland or in rabbits elsewhere.

6.4.3 ELISA tests for the presence of RHDV- and MYXV-specific antibodies

ELISA antibody tests were carried out on the 11 rabbit sera using both recombinant RHDV protein (Marin *et al.*, 1995, Moss *et al.*, 2002) and MYXV antigen (see methods). The results show that 10 out of the 11 sera contained detectable antibody to RHDV, with 9 of these showing antibody with a titre greater than 1:1280. These results confirm that a genetically divergent strain of RHDV does indeed circulate amongst rabbits, albeit harmlessly on Lambay Island, without causing overt disease. The ELISA results with MYXV showed 6 out of the 11 samples contained detectable antibody to MYXV, but at lower levels than the RHDV, suggesting that although there is MYXV circulating on the Island, the rabbits are mainly resistant to the virus. It must be stated that all the rabbits tested were young rabbits, and therefore may not have yet been exposed to MYXV. However, it is more likely that the absence of Myxomatosis despite the deliberate introduction of the disease indicates a level of genetic resistance within the rabbits. Thus the rabbits appear to be resistant to the Lambay Island strain of RHDV and MYXV. It will be interesting in the future to investigate the basis for this apparent dual resistance to two known highly pathogenic viruses.

6.4.4 Genetic divergence of Ashington, Lambay and RCV

The wide divergence of strains Ashington, Lambay and RCV indicates that these viruses must have emerged many years before the other strains of RHDV. However, of the three only Ashington has been associated with disease and this was observed to have occurred after 1984.

In view of the wide divergence of the Lambay Island lineage and its apparent isolation from other strains of RHDV, we decided to look for evidence of a genetic

link between RHDV and the European Brown Hare Syndrome virus (EBHSV). Based on comparative sequence alignments this virus was approximately 11% more divergent genetically from EBHSV (59% nucleotide identity), than it was from Ashington (69.1% nucleotide identity) or RCV (70.1% nucleotide identity).

Comparative deduced amino acid alignments between groups 1 and the other RHDV groups and also between group 1 and EBHSV were examined to see whether or not any significant amino acid sequences could be identified that might indicate genetic relationships not identified in the tree. There were no obvious motifs in Ashington, Lambay or RCV that could distinguish them from the other strains of RHDV or EBHSV. However, further analysis will be needed when the complete genomic sequences of these viruses become available.

6.4.5 Detection of recombination between two widely divergent strains of RHDV

Whilst preparing and analysing the phylogenetic tree described above it was noted that the relative topological position of a French isolate designated France 00-08, exhibited a very long branch length often indicative of recombination. Accordingly a sliding window analysis was performed in which this virus was tested for similarity against all other strains of RHDV. The results (Fig 6.3) confirm the conclusion based on tree incongruity, i.e. that the isolate France 00-08 is a recombinant virus with a predicted crossover point at nucleotide position 375 and 425 in the capsid genome. Moreover, the strain of virus most closely related to this region of the capsid genome was the strain Ashington.

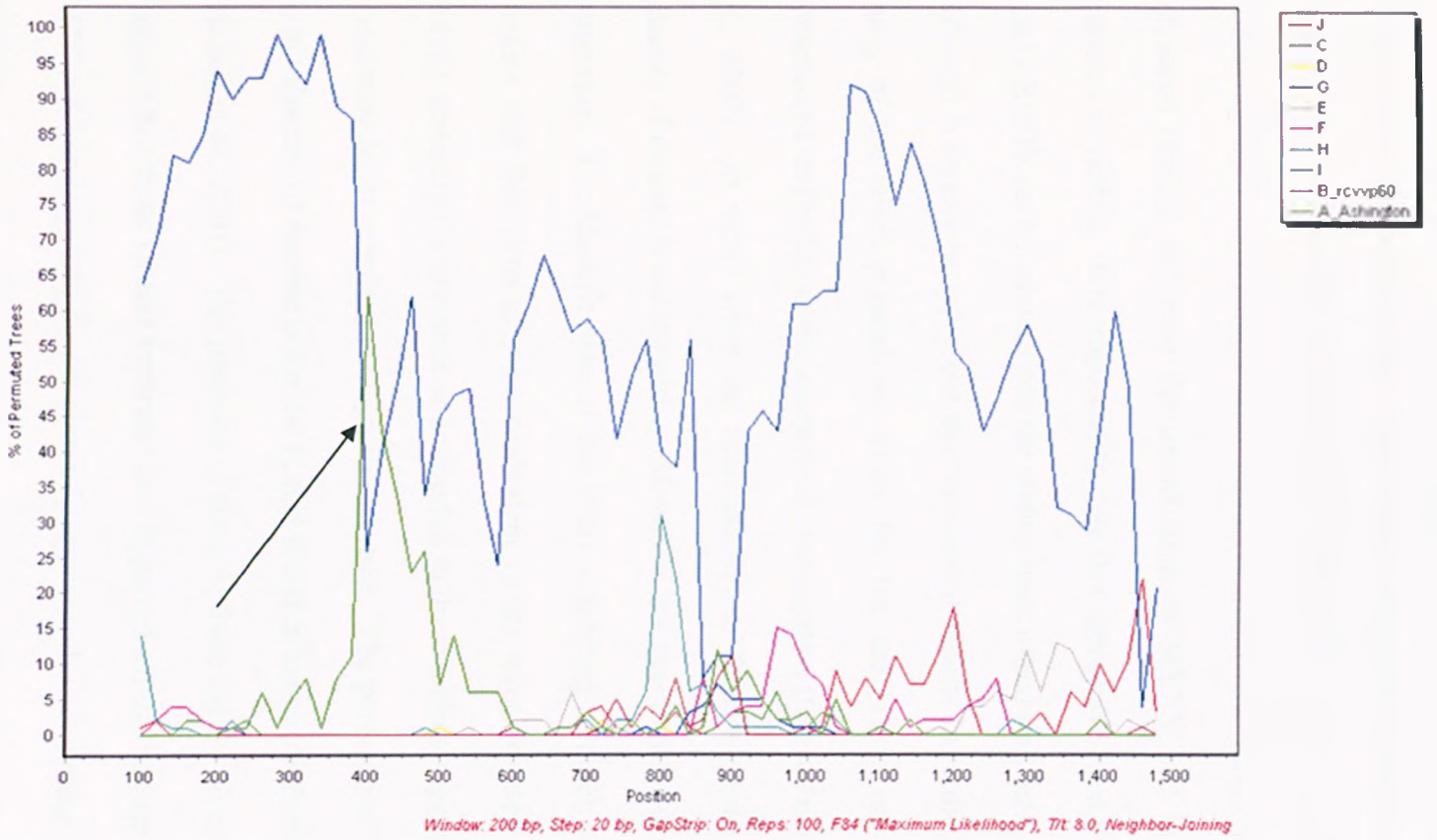


Figure 6.3
Bootscanning analysis of the France 00-08 strain against reference strains of RHDV. Showing evidence of recombination as indicated by the arrow. Groups are those groups identified in Chapter 4, with A and B representing strains Ashington and RCV, whilst C-J are groups 1-7

6.5 Discussion

The presence of the European rabbit on Lambay Island is probably the direct result of their introduction from the Dublin area approximately 20 years ago. This was prior to the identification of RHDV on the Irish mainland (Collery *et al.*, 1995). Following their introduction onto Lambay Island, the rabbit population density increased rapidly and even the introduction of MYXV had little impact on the numbers of rabbits. It is important to note that there have been no reports of deaths due to RHDV on the island since the rabbits were introduced more than 20 years ago, although it cannot be ruled out that occasional rabbits have died of RHDV. Like many of the rabbit populations within the UK the rabbits on Lambay show the presence of antibodies in the absence of overt disease (Chasey *et al.*, 1997a, Moss *et al.*, 2002). In many ways the identification of RHDV-specific sequence in the absence of disease is not surprising. However, the extent of the divergence was more surprising. The identification of this strain exhibiting as much divergence as RCV implies that this virus may be equivalent to the virus isolated by Capucci *et al.* (1996), certainly the presence of antibodies in these rabbits suggests that this virus is circulating as an avirulent contagious infection. The presence of viraemia in rabbits in the absence of disease is similar to that found in humans infected with Hepatitis G (Halasz *et al.*, 2001). The presence of three of these highly divergent RHDV strains suggests that these viruses separated and dispersed a long time ago, but in the case of RCV and Lambay, they do not currently appear to be circulating actively as virulent viruses.

The discovery of this lineage on Lambay Island suggests that there have been at least two introductions of RHDV onto mainland Ireland, the original lineage that gave rise to the Lambay strain, and a more recent introduction of a European lineage virus that has epidemiological characteristics more akin to those observed in the wetter regions of the world (Chapter 4). Recognising the limitations due to sampling error one can speculate that the Lambay strain has circulated in the Irish rabbit population but it may have been out-competed by more recently introduced strains, only surviving in isolated pockets. This is probably what has also happened for the Ashington strain and in effect these strains represent the equivalent of "living fossils". Interestingly, Ashington appears to have retained its ability to cause disease whereas we do not know if the viral RNA from Lambay can cause a fatal infection in a naïve rabbit, or whether it is like RCV, and causes only avirulent infections. On the basis of the phylogenetic evidence (Chapter 4) it appears that both Irish viruses were present in Ireland before the 1950's. It seems unlikely that an interaction between these two Irish viruses was responsible for the recent epidemic outbreaks that occurred in the 1990's, since there is no evidence of the Lambay virus in the regions from which the other Irish viruses were isolated and *vice versa*.

Interestingly, although strains Ashington and Lambay Island show a similar level of nucleotide identity to each other and to other strains of RHDV, strain Ashington shows a higher degree of similarity to EBHSV than Lambay. It is possible that the tree topology of Ashington, Lambay Island and RCV is skewed because of the lack of samples and the long branch lengths. Thus whilst it is believed that Ashington and Lambay are more closely related to each other rather than to RCV, this may change with the isolation of further strains in this group.

The identification of a French recombinant strain that exhibits an insertion/deletion mutation with a virus similar to Ashington is the first identification in RHDV of recombination between two disparate viruses. Since Ashington virus is not the strain involved in the recombination, it is likely that another virus of the same lineage or similar divergence as strain Ashington exists. Moreover the high degree of divergence between these two strains of RHDV (20%) would in another viral genera indicate the presence of heterologous recombination. It has been shown that recombination occurs in many other caliciviruses (Hardy *et al.*, 1997, Katayama *et al.*, 2002, Oliver *et al.*, 2004), although between viruses with greater homology than that demonstrated here. Further work may determine whether recombination between such disparate strains is common across the *Caliciviridae* or whether it only occurs in RHDV.

Clearly more studies are required before we will be able to explain why viruses that have apparently lain dormant for many years may suddenly reactivate. However, the Lambay Island strain of RHDV appears to be the most convincing thus far, that RHDV can survive long-term through the production of persistent infections.

Chapter 7

Recombination in *Rabbit haemorrhagic disease virus* (RHDV), a new perspective on its evolution and emergence

Manuscript in preparation

7.1 Summary

Emerging diseases cause significant mortality but it is far from clear why so many appear to be increasing in virulence and prevalence. The emergence of rabbit haemorrhagic disease (RHD) was first reported in 1984 following the introduction of apparently healthy rabbits into China from Germany. *Rabbit haemorrhagic disease virus* (RHDV) has since killed hundreds of millions of domestic and wild rabbits particularly in Europe, China and Australia. Our objectives are to understand the emergence and epidemiology of RHDV. Using comparative sequence data and phylogenies to look for tree congruency supported by bootscanning analysis, we present evidence of frequent recombination amongst strains of RHDV. Whilst this may be in part responsible for some epidemic outbreaks currently we have no direct evidence that it is the only potential mechanism by which RHDV may suddenly increase its virulence.

7.2 Introduction

Rabbit haemorrhagic disease virus (RHDV) is a highly virulent pathogen of the European rabbit (*Oryctolagus cuniculus*). It is a member of the genus *Lagovirus* in the family *Caliciviridae* (Ohlinger *et al.*, 1990, Ohlinger & Thiel, 1991), which contains positive single-stranded positive-sense RNA viruses. Rabbit haemorrhagic disease (RHD) disease was first described in China in 1984 where it rapidly killed an estimated 14 million farmed rabbits (Xu, 1991). The first recorded epidemic of RHDV in the UK occurred in 1992 in Southern England in domestic rabbits. Unlike the devastating epidemic in China, the virus did not spread rapidly throughout the domestic and wild populations in the UK, it caused small, localised epidemics that were patchy in their distribution and unpredictable in the number of rabbit fatalities (Chasey *et al.*, 1997a). The epidemiology of the virus in the UK is in stark contrast to that originally seen in France, Spain and Germany where, like China, the virus caused major epidemics killing large numbers of rabbits within short periods of time. In the UK, serological surveys have identified rabbits with RHDV-specific antibodies in every wild population sampled (Chasey *et al.*, 1997b, Moss *et al.*, 2002, Trout *et al.*, 1997b) and it has been suggested that this high level of immunity prevents the virus from killing large numbers of rabbits.

Retrospective analysis of sera collected before 1984 has shown that antibodies to the virus were present in both wild and domestic rabbits prior to the first recorded RHDV in, Czechoslovakia, the UK, New Zealand and Australia (Moss *et al.*, 2002, Nagesha *et al.*, 2000, Nowotny *et al.*, 1992, O'Keefe *et al.*, 1999, Rodak *et al.*, 1990). This suggests that RHDV or an antigenically similar virus circulated within the rabbit populations prior to 1984. This is also supported by the evidence that capsid sequence from rabbits that died following infection by RHDV was very

similar to sequence obtained from healthy rabbit sera, stored since 1955 (Moss *et al.*, 2002). It has also recently been proposed that persistent infections of RHDV developed in New Zealand rabbits following the deliberate introduction of virulent RHDV as a bio-control agent (Forrester *et al.*, 2003, Zheng *et al.*, 2000). This observation might explain how RHDV circulates amongst rabbits without causing overt infections, and it raises the important question, why did RHDV emerge as a highly virulent pathogen when it had apparently evolved a silent mode of circulation amongst rabbits? Whilst there are many possible explanations, one significant property of RHDV that has not yet been considered is investigated in this chapter, i.e. virus recombination.

As the result of improvements in computer programmes that perform comparative sequence analyses, homologous recombination has now been shown to occur commonly and quite frequently amongst many different families of positive stranded RNA viruses (Dahourou *et al.*, 2002, Santti *et al.*, 1999, Twiddy & Holmes, 2003, Weaver *et al.*, 1997). Whilst there is no direct evidence it is theoretically possible that the sudden appearance of highly virulent RHDV in China in 1984 and the other equivalent appearances of virulent RHDV in Europe described in this thesis might be explained by recombination. This chapter presents the results of the search for evidence of recombination within strains of RHDV.

7.3 Materials and Methods

7.3.1 Source of sequence data

Published sequences were downloaded from GenBank. Accession numbers and references can be found in table 7.1.

Sequence	Accession Number
German	NC_001543
Czech V351	U54983
Mexico	AF295785
SD	Z29514
Spain 89	Z24757
BS89	X87607
China CD	AY523410
Iowa	AF258618

Table 7.1
Sequences and their accession numbers downloaded from GenBank

7.3.2 Amplification of viral RNA using RT-PCR

Rabbit liver and serum samples containing RHDV and treated with RNAlater to preserve the RNA were obtained from Bahrain (Dr. Abubakr), Germany (Dr. Schirrmeier), New Zealand (Dr. Boag), Saudi Arabia (Dr. Abu Elzein) and the UK (Dr. Boag/Dr. Trout). For molecular analysis, the RNA was extracted from 100µl of serum (RNAlater treated) or 100µg of liver using the RNAgents kit (Promega) following the recommended protocols. Primer sequences for RT-PCR were designed using published RHDV data (Boga *et al.*, 1992, Gould *et al.*, 1997, Meyers *et al.*, 2000, Rasschaert *et al.*, 1995) and are described in Table 7.1. First strand cDNA synthesis was performed using Superscript II reverse transcriptase (Life Technologies) and the reverse external primer. The DNA was amplified by nested PCR, utilising the external and internal primers described in Table 3.2 (p66) yielding seven products of between 600 and 1750bp. The resulting products were gel purified and both strands were sequenced using a PE Biosystems cycle sequencing kit with the sequencing primers producing approximately 500bp of sequence for analysis.

The sequences were assembled using Pregap4 and Gap4 (Staden Package), subsequent analysis was carried out using Translate (GCG, Wisconsin Package) and jEmboss. The full length genomes were aligned using ClustalX (Thompson *et al.*, 1997), and further analysis was carried out using BioEdit (Hall, 1999).

7.3.3 Analysis to determine genetic recombination

Evidence for recombination was investigated using the SIMPLOT program version 3.5.1 (Lole *et al.*, 1999, Ray, 1997), with bootscanning analysis (Salminen *et al.*, 1995). During the bootscanning procedure, the alignment was divided into sequential segments of 200 nt, overlapping every previous segment by 180 nt (to produce a window width of 20 nt). A bootstrapped analysis using 100 replicates was applied to each segment by using the Neighbour-Joining algorithm, with Maximum Likelihood settings and a transition/transversion ratio as determined by MODELTEST 3.06 (Posada & Crandall, 1998). The sequence under investigation was compared against reference sequences and the probability of the sequence falling within a specified group was estimated along the genome. Only bootstrap scores of >70% indicate significant support (Felsenstein, 1985).

To support the SIMPLOT results, phylogenetic trees were constructed for each of the genomic regions, nt 0-5300 and 5301- 7434. Phylogenetic analyses were carried out using PAUP* version 4.0,10b (Swofford, 2000). The optimal model for each dataset was estimated using MODELTEST v3.06 (Posada & Crandall, 1998). The optimal models (TrNef+Γ, and TrNef+I+Γ) were then used to estimate the Maximum Likelihood tree using iterative heuristic searches with TBR branch swapping algorithms with re-estimation of variable parameters between searches, from the data, where necessary. A neighbor-joining tree was also estimated using the optimal Maximum Likelihood model and settings. In every case neighbour-joining

trees were congruent with the Maximum Likelihood trees. Neighbor-joining bootstrap support (1000 replicates) was calculated for each tree using the Maximum Likelihood settings.

7.4 Results

A bootscanning sliding window analysis of the 25 sequences in the database identified ten potential recombinant viruses. The regions of each genome sequence involved in recombination and the predicted site at which crossover of the polymerase occurred are identified in Table 7.2. Three viruses showing evidence of recombination, namely Frankfurt 5, Frankfurt 12 and Wika appeared to be direct descendants of a single recombination event. All three of these viruses group together on the phylogenetic tree (see Fig 7.1b) and show significant homology to each other, Frankfurt 5 and Frankfurt 12 are the same virus sequentially passaged in different rabbits, and Wika is a strain from a wild rabbit isolated from the same region as the Frankfurt isolate. These isolates are very similar making it difficult to determine which is descended from which virus. These three recombinant viruses occupy branches in the phylogenetic tree that are basal to the lineage represented by the China 1984 virus. Therefore recombination in the parent viruses occurred before the first recognized outbreak of RHDV in 1984 (Liu *et al.*, 1984).

Sequence	1st Crossover (nt)	2nd Crossover (nt)	Region of Genome
Frankfurt5	4125	4500	RdRp Polymerase
Frankfurt12	4125	4500	RdRp Polymerase
Wika	4125	4500	RdRp Polymerase
Czech	2000 6125	2500 6375	p37/29 (see Fig 1.1) Capsid protein
German	2000	2500	p37/29
NZ54	6125	6375	Capsid protein
NZ61	6125	6375	Capsid protein
Hagenow	5300		Capsid protein
Hartmannsdorf	5750 6875	5875	Capsid protein Capsid protein
Mexico	5300		Capsid protein

Table 7.2
Showing the number and position of identifiable crossover points of the polymerase leading to recombination in 10 strains of RHDV

The Czech v351 and German strain (see Fig 7.2) are also grouped together in the phylogenetic tree and they share a common region of the genome in which a double crossover of the RNA polymerase enzyme has occurred (nt 2000 and 2500). On the basis of the phylogeny (Fig. 7.1b) recombination appears to have occurred in an ancestral lineage of these two viruses. However, it is important to note that the Chinese 1984 strain had already separated from this lineage. This is also supported by the observation that no other direct descendants of the Chinese 1984 lineage, viz. the Mexican isolate or the Saudi Arabian isolate show evidence of this particular recombination. The Czech virus differs from the German virus in that there is a dual crossover point in the capsid protein occurring at nt 6125 and 6500. Since the descendant German isolate does not show evidence of recombination in this region of the capsid protein, it presumably occurred after the two had separated.

Interestingly, the New Zealand strains, NZ54 and NZ61, the lineages of which are directly descended from the Czech strain shows evidence of only one of the double crossover points found in the Czech strain, namely nt 6125 and 6500. Whilst there is some evidence within these genomes for the other crossover point at nt 2000 and 2500, this is not as statistically robust as that within the Czech strain, suggesting that subsequent mutations have masked the evidence for this crossover. This implies that there may be many more instances of recombination within the strains of RHDV that subsequently been masked by the accumulation of point mutations.

Recombination was also detected within the capsid gene, between nucleotides 5305-7044 in strains Mexico, Hartmannsdorf and Hagenow. Of all the recombinant strains, Hagenow shows probably the most pronounced phenotypic consequence of recombination with a single crossover. This occurred at the beginning of the capsid gene at nucleotide 5300 (Fig. 7.1a). Thus, from the 5' end of the genome until the capsid gene, the strain Hagenow is most closely related to strain Hartmannsdorf (Fig. 7.1bi), but after the capsid gene (nt 5305-7044) it becomes basal to three of the groups, e.g. the viruses Meiningen, Ascot, BS89 and the Bahrain and Jena group (Fig. 7.1bii). It is not possible to determine at which stage of evolution this recombinant virus emerged. However, the lineages that gave rise to the capsid region of strain Hagenow probably date a long time prior to 1984. The level of "background noise" present in the 5' end of the genome for the strain Hagenow indicates that one of the strains involved in the recombination has not been identified. The strain Mexico shows the same evidence of a single crossover point at the beginning of the capsid gene as strain Hagenow. However, the two strains between which the recombination occurred appear to be closely related as only a very small alteration in tree topology can be observed for this virus (see Fig. 7.1b).

Three regions where recombination occurred have so far been identified. Firstly, the region of the viral genome that incorporates nt 2000-2500 includes the protein cleavage site from which the helicase protein (p37) and another protein of

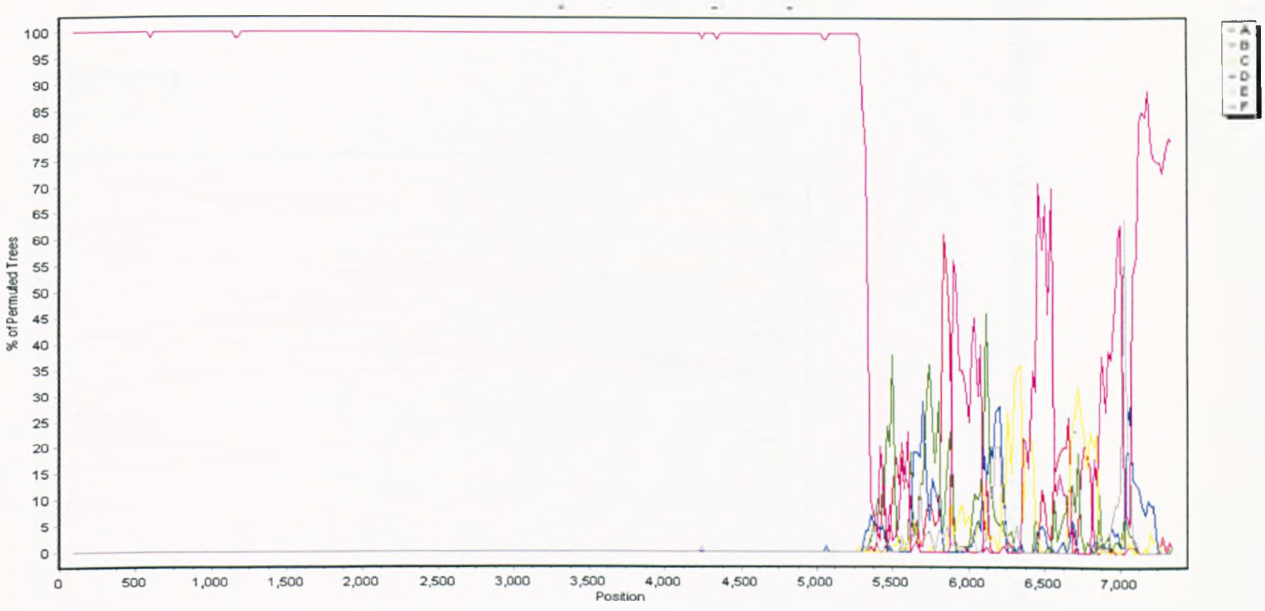
unknown function (p29) are derived during post-translational processing. The second predicted hotspot for recombination involves nt 5300 - 7044, which corresponds to the capsid protein gene. Within this region, a highly variable domain has been identified from nt 6125-6500. Although several examples of recombination have been identified further comparative analyses will need to be undertaken to determine the level of inherent variation amongst these strains.

A secondary analysis of a database of the capsid gene (see Chapter 5) was also carried out, to look for evidence of recombination in the Chinese 1984 sequence. This analysis was carried out as only the capsid gene of the Chinese 1984 strain has been sequenced and therefore the alignment of the capsid gene used in Chapter 5 was used for a secondary analysis. Evidence of recombination was detected in the capsid sequence of the Chinese 1984 strain as indicated in Figure 7.3. However, it must be stated that whilst there is evidence for recombination, the analysis suggests that the virus that gave rise to the recombinant strain has not yet been identified and this would account for the high background noise present in the first half of the capsid protein.

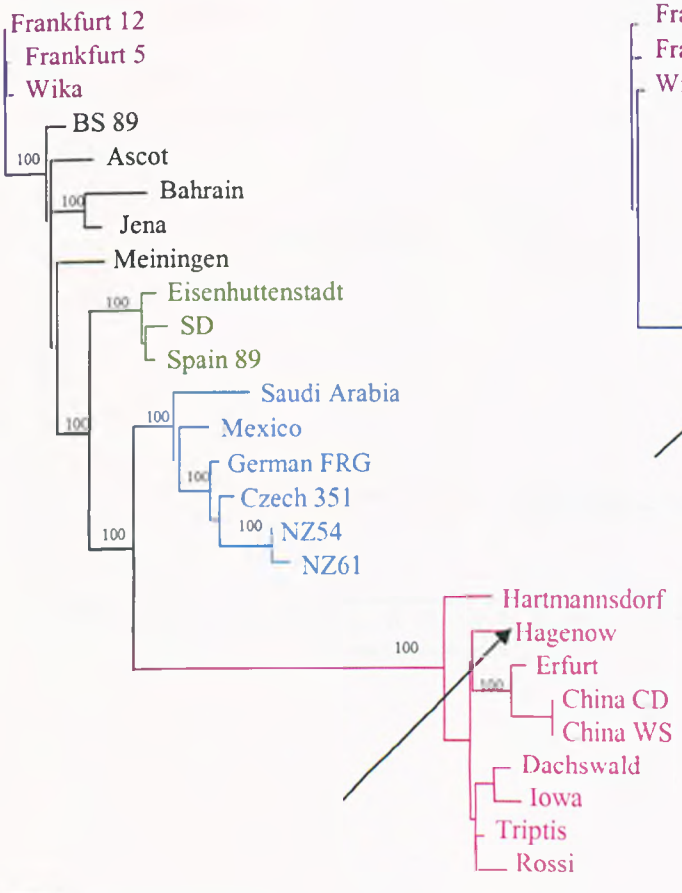
Figure 7.1

Showing the sliding window analysis for strain Hagenow, with the recombination point at 5300 nucleotides (Fig. 7.1a) and corresponding phylogenetic trees (Fig. 7.1b). The groups correspond to previous groups as indicated in Chapter 4, where A-D indicate groups 1-4, groups 5 and 6 were grouped together as E for the purpose of this analysis, and group 7 is represented by H. The phylogenetic trees show the difference in tree topologies for strain Hagenow when nt 0-5300 are considered (bi) and when nt 5301-7044 are considered (bii), as indicated by the arrows. Phylogenetic analysis of the two datasets described above (see Methods) for 25 strains of RHDV. Maximum Likelihood phylogeny was calculated using PAUP* version 4.0b10. The optimal models to use with the data for (bi) was (TrNef+Γ), and for (bii) was (TrNef+I+Γ) were determined using MODELTEST 3.06, and the variable parameters were estimated from the data. Bootstrap values (shown only on the major branches, for clarity) were estimated for both trees using the Neighbour-Joining algorithm under the Maximum Likelihood model for 1000 replicates. Both Maximum Likelihood and Neighbour-Joining trees were congruent.

(a)



(*bi*)



(*bii*)

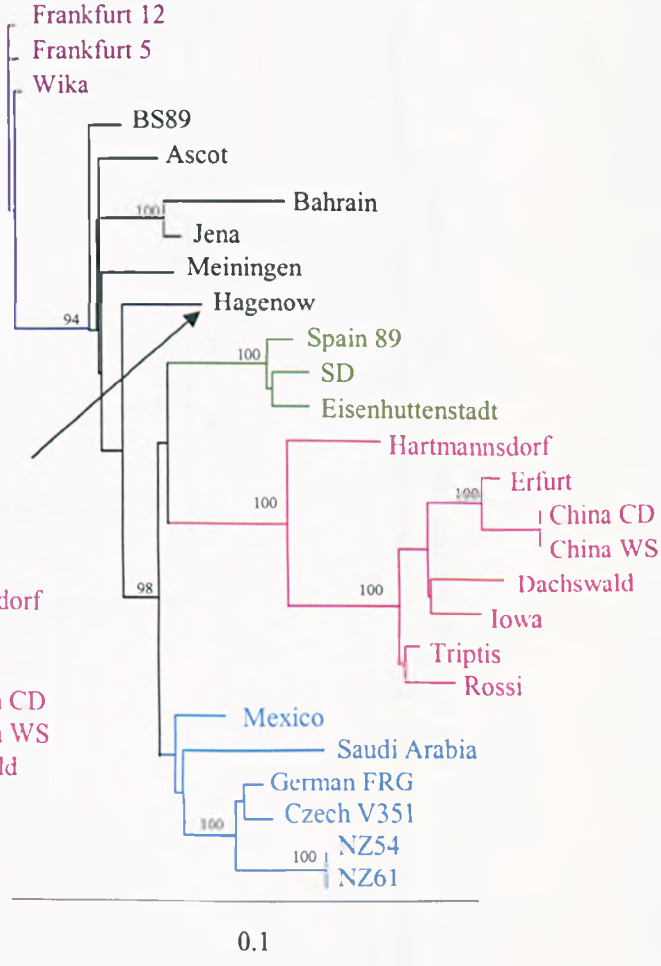


Figure 7.1

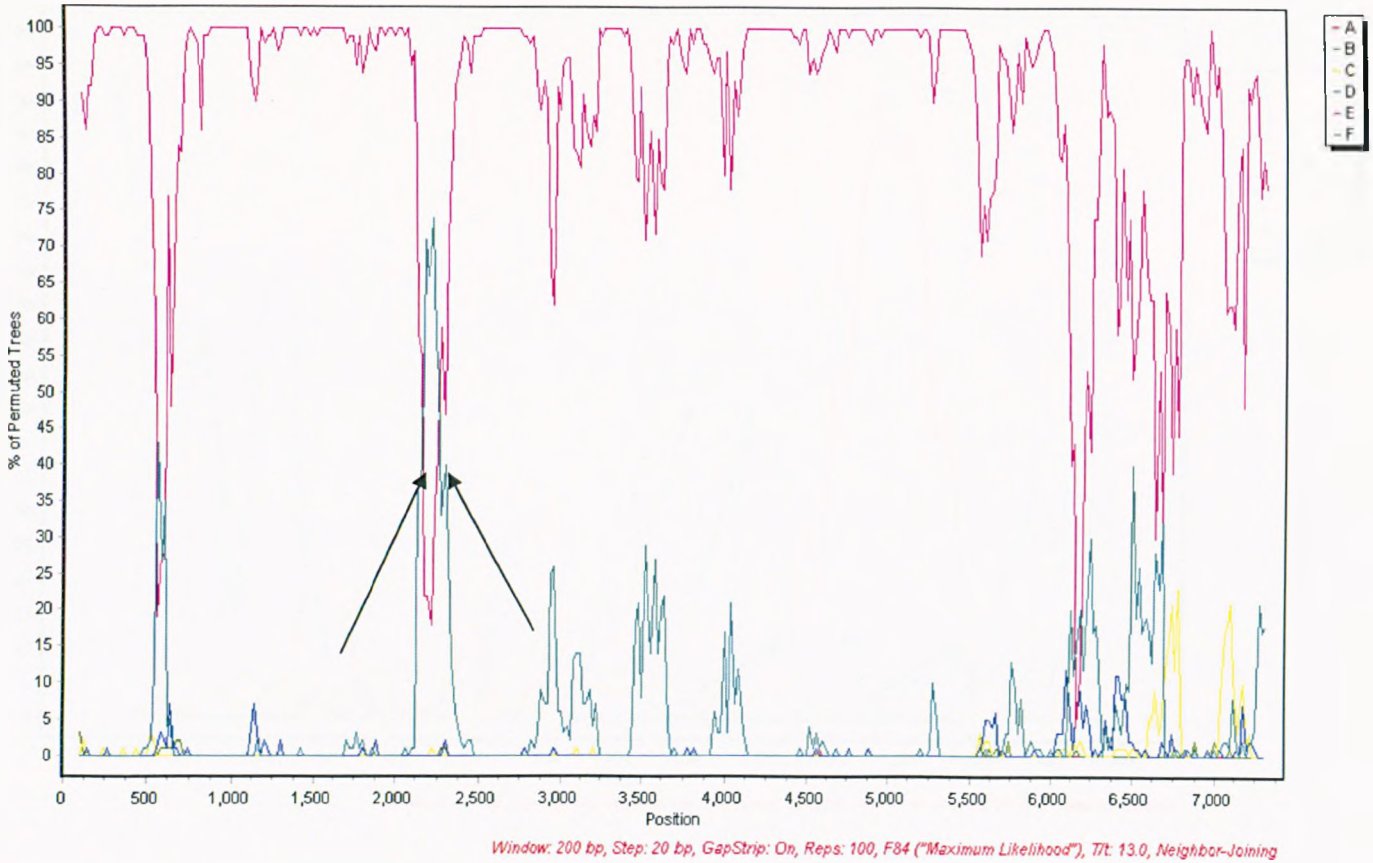


Figure 7.2
Bootscanning analysis of the German FRG strain of RHDV against reference sequences of RHDV. The crossover event is indicated by the arrows, groups are the same as for the Hagenow strain (see above)

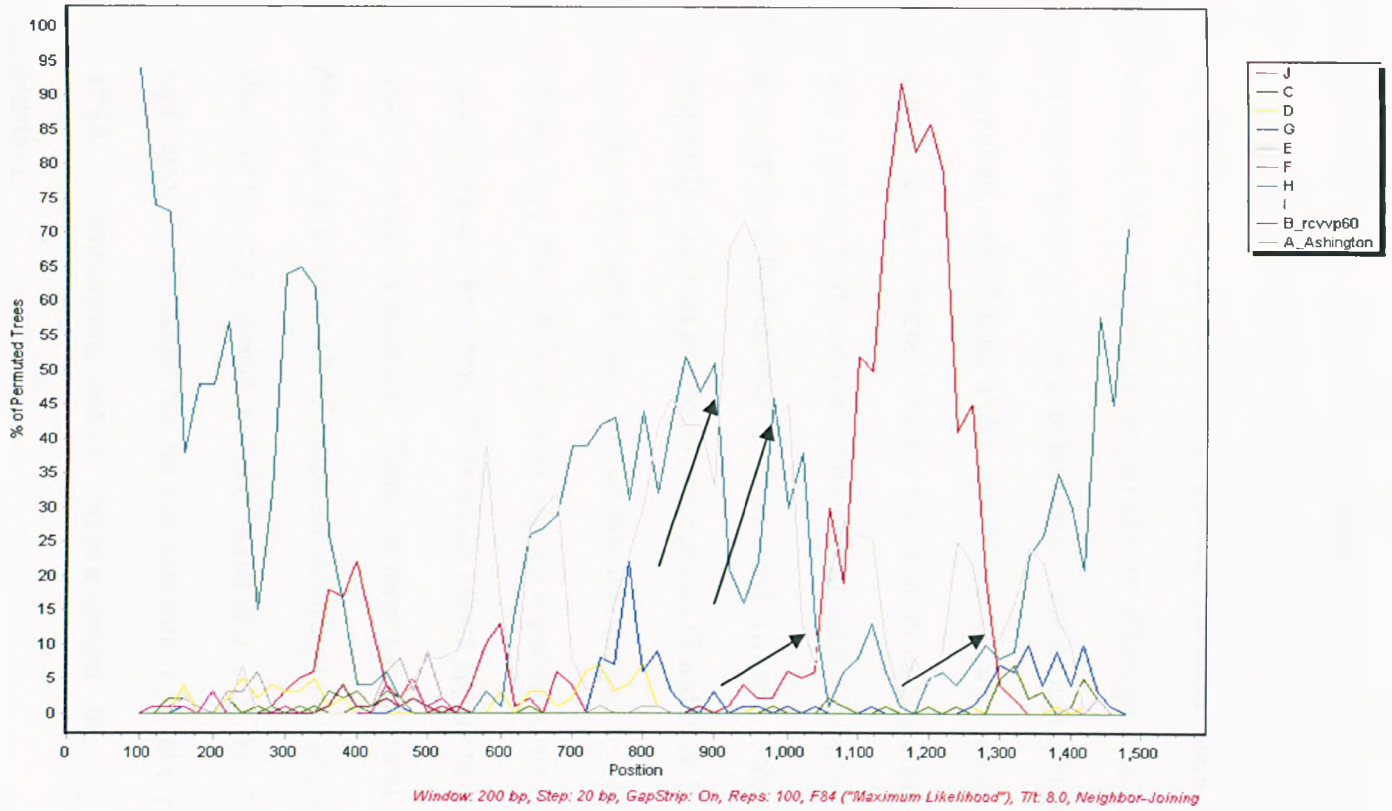


Figure 7.3
Bootscanning analysis of the Chinese 1984 strain against reference strains of RHDV. Showing evidence of recombination as indicated by the arrows. Groups are as the groups identified in Chapter 4, with A and B representing strains Ashington and RCV, whilst C-J correspond to groups 1-7.

7.5 Discussion

Compared with DNA viruses, genetic variation due to mutation in RNA viruses occurs with a much higher frequency mainly because of the lack of RNA polymerase proofreading activity during RNA transcription (Steinhauer *et al.*, 1992). The extent of the contribution to genetic variation made by recombination in positive-sense RNA viruses is not yet known although it is clearly potentially very important as a factor in virus pathogenesis. This is highlighted by the appearance of the recombinant encephalitic alphavirus *Western equine encephalitis virus* (WEEV), the apparent result of recombination between an encephalitis-inducing New World virus and a polyarthritis-inducing Old World virus (Hahn *et al.*, 1988). It is now becoming apparent that at least in some RNA viruses, recombination occurs much more frequently than was previously recognised (Twiddy & Holmes, 2003). Indeed, the rate of recombination may be variable and unique to each virus species, some recombining very frequently, such as *Murine hepatitis virus* (MHV) (Banner & Lai, 1991) and TT virus (Worobey, 2000) whilst others appear to be constrained showing little evidence of recombination. There are three hypothetical mechanisms by which recombination may occur; all of which are described in Nagy and Simon (1997), and all conform to the copy-choice model (Cooper *et al.*, 1974). All mechanisms require virus replication to be occurring for recombination to take place, which contrasts with DNA recombination where enzyme-driven breakage joining facilitates recombination.

There are certain prerequisites for recombination amongst RNA viruses (Worobey & Holmes, 1999), not least the co-infection of a single cell, replication in the presence of another strain of viral RNA and genetic compatibility between the recombinant molecules. Each of these conditions is likely to be satisfied by RHDV.

Firstly, it has recently been demonstrated (Forrester *et al.*, 2003) that RHDV establishes persistence in healthy rabbits, since entire genomes in the form of RNA can be extracted from the liver of healthy rabbits, providing ideal conditions for recombination with a superinfecting virus, although it is unknown if this RNA represents infectious virus. At the present time only infectious viruses isolated from dead rabbits have been shown to exhibit recombination (see below). However, this probably reflects, the small number of full-length viral RNA sequences analysed from healthy rabbits. Secondly, the extent of genetic variation between many strains of RHDV is relatively small, thus ensuring a high likelihood of compatibility that might be expected to favour recombination. There are three possible methods by which recombination may occur in RHDV. Firstly, as we do not yet know the degree of variation within populations it is possible that within a single infection quasispecies may be generated, and the presence of these quasispecies may lead to recombination events between variants of a single infection (Bello *et al.*, 2004). Secondly, a healthy rabbit infected with a persistent strain of RHDV may be secondarily infected by a more virulent strain that is then able to recombine with the resident persistently infecting strain. Hypothetically this could explain why certain strains in the UK showed both virulent and avirulent phenotypes in the absence of genetic change (Moss *et al.*, 2002). Thirdly, two viruses capable of inducing persistent infections may co-infect a single rabbit, providing a long-term opportunity for recombination.

On the basis of evidence provided in this chapter recombination appears to occur quite readily in RHDV genomes with 10 out of the 25 (40%) full-length genomes showing evidence of recombination. Two other strains of RHDV for which only capsid sequences are available have also been identified as recombinants,

(France 00-08 (Chapter 6) and China 1984). Interestingly the type of recombination seen most frequently thus far is insertion of a fragment of approximately 500 bp (see Fig. 7.2). Nevertheless, the presence of a single crossover point was also found in RHDV genomes as shown by strains Hagenow and Mexico. This single crossover occurred at the point at which the capsid gene is cleaved from the rest of the proteins. Further analysis will reveal whether or not recombination is confined to specific areas within the genome. Interestingly, whilst recombination has been shown to occur within other caliciviruses, namely Noroviruses (Hardy, *et al.*, 1997, Katayama *et al.*, 2002, Oliver *et al.*, 2004) all recombination in these viruses to date has been shown to occur between the ORF1 and ORF2 (with ORF2 analogous to the capsid protein in RHDV). Further work will need to be done to determine whether this difference is due to the variation in genome organisation or simply an artefact of the different methods used to determine the presence of recombination. However, it cannot be ruled out that the presence of the extra ORF in other caliciviruses may mean that recombination occurs more frequently between ORF1 and ORF2 resulting in a bias in reporting compared to RHDV.

Whilst there is no evidence to date, there is the possibility that recombination between two different strains of RHDV could explain why virulent virus appears to have arisen from persistent strains of RHDV. For example, the presence of recombination within the capsid gene of the Chinese 1984 strain (Fig. 7.3) might be responsible for a change in virulence that led to the initial outbreak in China in 1984. This is not unprecedented since recombination between two strains of Tomato yellow leaf curl virus led to an increase in virulence of these RNA viruses (Monci *et al.*, 2002). Two insertions were identified in the recombinant Chinese 1984 strain, and both show high bootstrap support. However, whilst recombination could be

responsible for the initial recorded epidemic outbreak, the probability of recombination being responsible for all the other epidemic outbreaks, that are predicted to have occurred in this thesis, is small. Many of the other epidemic viruses that occupy different lineages from the Chinese 1984 lineage have not been shown to be recombinants. Clearly the role of recombination and its significance in the genetic variation, and virulence of RHDV needs to be assessed through more extensive studies.

Chapter 8

A novel mechanism by which RHDV may survive long-term in the European rabbit; the presence of RHDV-like sequence in the DNA of rabbit liver cells

8.1 Summary

Although RHDV is a highly virulent disease of rabbits, it appears that the virus is also able to survive long-term within the rabbit in an apparently avirulent or persistent form. Recent work has demonstrated that the RNA of several non-retro-RNA viruses may be reverse-transcribed to produce cDNA that exists in infected cells, either integrated into cellular genomes or alternatively in an episomal form. This strategy may provide a mechanism by which RHDV could survive long-term in the European rabbit. Preliminary results are presented to show evidence of the presence of RHDV-like sequences in the DNA extracted from rabbit liver obtained from an animal that died following infection with RHDV, and from the liver of a healthy rabbit. This may have significant implications for the understanding of RHDV immunopathology and long-term survival.

8.2 Introduction

Recent studies on archival rabbit sera collected from healthy rabbits as far back as the mid 1950's (Moss *et al.*, 2002) showed that a high proportion of the sera contained RHDV-specific antibodies and some also contained detectable RHDV-specific fragments. Moreover, the sequence of these viral RNA fragments was almost identical to that of extant viral sequence isolated from rabbits that died of rabbit haemorrhagic disease (RHD) in the UK in the late 1990's. In addition it was recently shown that virulent RHDV, deliberately introduced into wild rabbit populations, can readily establish avirulent infections in wild rabbits (Forrester *et al.*, 2003, Zheng *et al.*, 2002), and that the sequence of the viral RNA recovered from these healthy rabbits closely resembled that of the viruses that caused lethal infections.

It has also been recently demonstrated (Chapter 4) that the lineages of RHDV are far more divergent than can be explained by genetic variation within the last 20 years, i.e. the period of time since the first recognised fatal epidemic outbreak of RHDV. It is also clear that the virus survives for long periods of time within the environment causing only periodic outbreaks with no evidence of disease between epidemics (Henning, 2003) However, as yet there is no adequate explanation for how RHDV persists long-term without necessarily causing disease.

Whilst several possibilities immediately spring to mind such as persistent infections, latency, long-term virus stability, membrane stabilisation etc., all of which need to be investigated thoroughly, we have considered an alternative possibility based on accumulating evidence for the presence of cDNA in cells resembling viral sequences (Crochu *et al.*, 2004, Drynov *et al.*, 1981, Gaidamovich *et al.*, 1978,

Klenerman *et al.*, 1997, Zhdanov, 1975, Zhdanov & Azadova, 1976, Zhdanov & Pafanovich, 1974). This chapter describes preliminary attempts to look for evidence of cDNA fragments corresponding to RHDV RNA, in liver taken from rabbits known to contain genomic length virus-specific RNA.

8.3 Materials and Methods

8.3.1 DNA extraction

Three liver fragments collected in New Zealand and the UK, from apparently healthy rabbits, or from rabbits known to have died following infection with RHDV were incubated in the denaturing solution from the RNAgents extraction kit (Promega) for 24-48 hours at 4°C. DNA extraction was then performed using an RNAgents extraction kit (Promega) following the manufacturer's instructions, but substituting 600µl of Phenol:Chloroform:Isoamyl Alcohol (25:24:1, v/v) (Invitrogen) at the phenol stage to precipitate DNA rather than RNA.

8.3.2 Digestion by nucleases

DNA extracted from the livers of rabbits was digested at 37°C for 2 hours using 10µg of RNAase A ml^{-1} (Gibco).

8.3.3 Amplification of DNA by PCR

In order to maximise the chance of detecting integrated RHDV sequence, the entire genome was scanned with each primer set designed to amplify ~500 bp of sequence. A nested PCR was carried out, with the first-round primers amplifying approximately 1000bp and second-round primers amplifying approximately 500bp. Both first- and second-round products were visualised on a 1% agarose gel using

electrophoresis. All clear bands were excised individually and purified using the Quiagen gel extraction kit. The resulting DNA products were then sequenced using the primers that had been used to produce the PCR products.

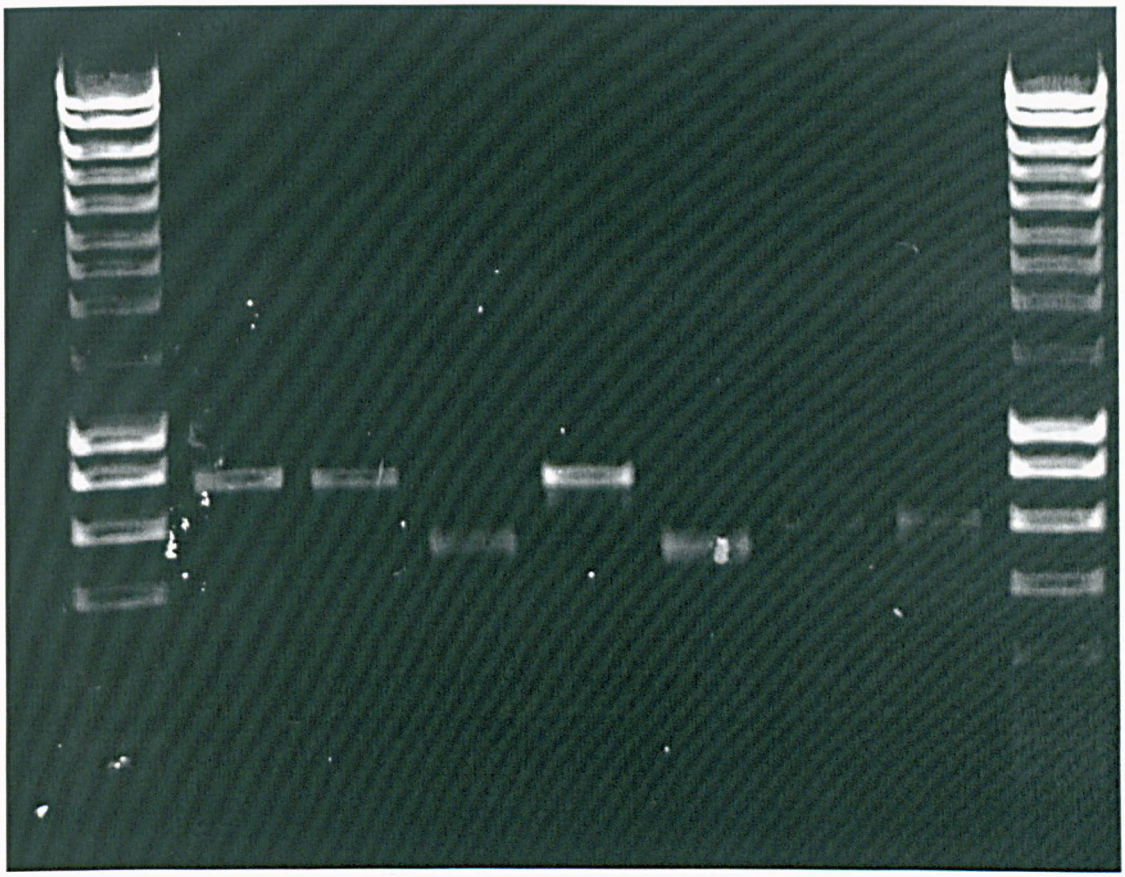


Figure 8.1
Showing the variation in band size produced from RT-PCR amplification of Rabbit DNA when primers amplifying 500bp of RHDV were used

8.3.4 Sequence Analysis

All derived sequences were compared with known RHDV sequence. The identity of sequence not dissimilar to RHDV was assessed using the Basic Local Alignment Search Tool (Altschul *et al.*, 1990) implemented via the National Centre

for Biotechnology Information website (www.ncbi.nih.gov/blast/) against the complete GenBank database. The BLASTN (nucleotide query – nucleotide database comparison) and BLASTP (protein query – protein database comparison) algorithms were used.

8.4 Results

DNA extracted from known RHDV-positive dead or healthy rabbits and a known RNA negative rabbit liver as determined by nested RT-PCR (Forrester *et al.*, 2003), was subjected to PCR and analysed as described in Methods. DNA sequence was obtained from fragments amplified using known RHDV primers (RHDV 5862F & RHDV 6234R, RHDV6135F & RHDV 6700R) coding for approximately 500bp of RHDV sequence (Fig. 8.1). Amongst the many sequences obtained, two contained nucleotide regions of approximately 70bp with sequence showing about 60% homology to the RHDV capsid protein region, but both sequences corresponded to different parts of the capsid protein. The first sequence corresponded to nucleotides (nt) 74-122 of the capsid sequence and the second to nt 317-388 of the capsid sequence (Fig. 8.2). These regions showed approximately 60.5% and 62.5% homology respectively with known RHDV sequences. It is important to note that 60% homology with known RHDV sequences is equivalent to the level of homology reported previously between flavivirus sequences and DNA from mosquito genomes (Crochu *et al.*, 2004). Within both sequences there was a region of approximately 24 bp that was characteristic of a mammalian genome. Although this sequence was very short, homology was high along the short length sequence, it must be stated that the probability of this occurring randomly is high (p=2.5). However, it is included for information as one of the homologous regions was part of the human complement

gene, and this could have a significant bearing on the pathology of RHDV. Contiguous with the RHDV-like DNA sequence was coding that was not homologous to RHDV, and a BLASTN search revealed that the flanking sequences adjacent to this putative RHDV-like sequence were homologous to a mouse (*Mus musculus*) gene and a chimpanzee (*Pan troglodytes*) gene (Fig. 8.2). These differing flanking sequences were found at both the 5' and 3' ends of the RHDV-like sequence. The presence of these flanking sequences showing no homology to RHDV ruled out the possibility that viral RNA had been inadvertently sequenced.

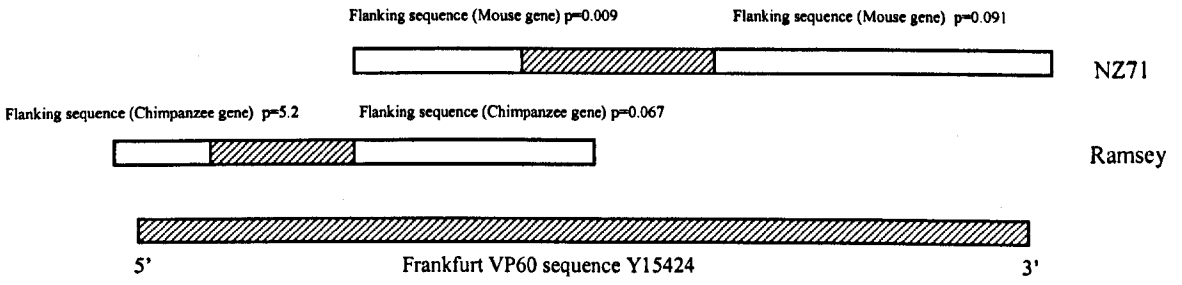


Figure 8.2 Showing regions of homology between the isolated DNA sequences and a reference sequence. Hatched regions indicate the region homologous to RHDV. Flanking sequence with the BLAST E-value are also shown

Thus far there has been very little sequencing of the *O. cuniculus* genome, and therefore it can only be assumed that the similarity reported here to mammalian genes indicates the presence of *O. cuniculus* sequence. Further analysis will be required to confirm the type and region of the genome into which these viral sequences have apparently integrated. In addition, more DNA sequences will need to be identified before it is possible to attach a meaningful significance to these results.

8.5 Discussion

Recent studies on the origin and evolution of RHDV suggest that the virus has been circulating amongst rabbits, probably harmlessly for many years prior to the first epidemic reported in 1984. Whilst it is possible that RHDV did cause minor outbreaks of fatal disease in rabbits prior to 1984, they were clearly missed by all observers in Europe if indeed they did occur. This lack of evidence for epidemic RHDV in the literature prior to 1984 has inevitably raised the possibility that RHDV could have maintained an infectious cycle in rabbits without causing disease. However, there has been no formal demonstration of anything resembling a persistent infection, although an attenuated strain of RHDV, designated Rabbit calicivirus (RCV) was isolated in domestic rabbits in Italy (Capucci *et al.*, 1996b). Moreover, there have been several outbreaks of RHDV in North America, Mexico and the Caribbean, the precise sources of which have never been adequately explained. Apart from the concept of a persistent infection an alternative possibility is that during the long-term symbiosis between rabbits and RHDV, the viral RNA has evolved the capacity to be reverse-transcribed and integrated into the rabbit DNA either in the genome or as episomal DNA.

This suggestion prompted a search for evidence of RHDV-like DNA in rabbit liver. Following examination of the entire genome of RHDV two small fragments of rabbit liver cell DNA were obtained that were 60% homologous with RHDV RNA. Each of these fragments aligned with a different region of the capsid protein, which is found at the 3' end of the RHDV genome. Since the two fragments were both identified as capsid protein it is possible that only the sub-genomic RNA species is capable of integration. Clearly more studies are required before this possibility can be assessed properly. Both DNA fragments possessed flanking regions at the 5' end

and the 3'end with the RHDV-like sequence situated between these flanking sequences. There are two possibilities to explain this. Firstly, these fragments may represent remnants of integration events into the genome that have happened at least twice and possibly more often. Further work is required to determine if these fragments are remnants of a larger integration event that has been slowly lost over a long period of time. The possibility of these fragments representing recent lineages seems unlikely considering the homology difference between the fragment and the extant RHDV genome. Although this degree of homology (60%) is less than that found between even the least closely related extant RHDV strains, three isolates of RHDV have been identified showing no more than 80% homology to the other strains of RHDV and also to each other (Ashington, RCV and Lambay – see Chapter 6). These strains are the most divergent and therefore show a more ancient lineage than all other RHDV strains. It is possible that the 60% homology of the DNA fragments represents the integration of an ancestral lineage of these three strains.

Secondly, it is possible that these regions of homology are the result of viral mimicry. It is well known that mimicry by a virus can permit viral antigen binding to the host cells (Barnett & Fujinami, 1992, Falconar, 1997, Oldstone, 1987) and if this occurs in the vascular tissue may result in haemorrhagic pathology such as that seen in Dengue haemorrhagic fever and RHDV (Chang *et al.*, 2002, Halstead, 1989). Moreover, for example the disruption of vascular tissue during human Ebola virus infection only occurs when macrophages are present along the vascular lining (Ryabchikova & Price, 2004), suggesting than an autoimmune reaction contributes to the observed pathology. The genome of *O. cuniculus* has not yet been sequenced, but further work may identify potential cross-reactive epitopes that elicit host autoimmune reactions that culminate in haemorrhagic manifestations. However, this

may also explain why, in some rabbits, the virus is able to kill the infected host even in the presence of high levels of antibody (Calvete *et al.*, 2002, Marchandeau *et al.*, 2005).

As yet this work has raised more questions than it has answered. Further studies should determine whether the observed RHDV-homologous DNA in the liver of healthy rabbits represents viral RNA transcribed to DNA or alternatively represents random sequence with shared domains. These studies will lead to a significant step forward in our understanding of RHDV persistence and if the viral mimicry appears to be correct may shed light on the immunopathology of the infection.

Chapter 9

Myxomatosis and RHDV studied in free-living rabbits in the UK

9.1 Summary

The presence of the Myxoma virus (MYXV) in populations of rabbits infected with RHDV may influence the impact of RHDV on the rabbit population and *vice versa*. A long-term study of the antibody status of recaptured rabbits from three free-living populations of wild rabbits was undertaken. The antibody status of each rabbit was determined by ELISA for both RHDV and MYXV and the long-term antibody status of the three populations was compared. In two of the populations antibody levels against RHDV and MYXV showed consistently high levels suggesting that both viruses were circulating concurrently within these rabbit populations. The population at the third site, Exminster showed a marked change in proportion of both RHDV and MYXV antibodies over the three years with RHDV seroprevalence increasing and MYXV seroprevalence decreasing during the second year. It is possible that this represents one disease suppressing the circulation of a second virus. This may have serious implications for bio-control of rabbits where two viruses are circulating concurrently within the population. Models were used to estimate the level of interaction, if any, between RHDV and MYXV. No obvious interactions were observed suggesting that RHDV and MYXV probably circulate independently of each other.

9.2 Introduction

In the past fifty years the wild European rabbit (*Oryctolagus cuniculus*) populations in the UK have been devastated by two major viral pathogens. Myxoma virus (MYXV) was introduced into the UK in 1953 (Armour & Thompson, 1955). Rabbit haemorrhagic disease virus (RHDV) was observed to cause fatal epidemic outbreaks for the first time in wild UK rabbit populations in 1994, although recent evidence suggests that the virus had been circulating in the absence of disease prior to 1994 (Moss *et al.*, 2002). In both cases the appearance of the virus caused significant reduction in the number of rabbits but to date there have been no investigations to see whether or not interactions between these viruses have occurred and what impact this might have had on the rabbit populations.

9.2.1 Myxoma virus

In the wild, MYXV naturally infects the Brazilian tapeti (*Sylvilagus brasiliensis*) (Aragão, 1943), and the Californian Brush Rabbit (*S. bachmani*) (Marshall & Regnery, 1960) causing the development of a small fibroma on the ear, but no significant morbidity or mortality. It was first recognised in 1898 that under experimental conditions the virus causes a highly virulent infection of domestic rabbits (Sanarelli, 1898) thus making it a potential bio-control agent. However, although trials were carried out to test its efficacy as a method for rabbit control, in Europe and Australia, the disease did not become established in wild rabbit populations until the late 1940's (Fenner & Ross, 1994). In 1953 a MYXV-infected rabbit was smuggled into the UK and by 1955 the virus had dispersed throughout the UK rabbit populations (Armour & Thompson, 1955). It has been estimated that the initial outbreak killed 99% of wild rabbits (Brown *et al.*, 1956, Hudson *et al.*, 1955).

However, within three years, low virulence strains of MYXV were being isolated from wild rabbits (Hudson *et al.*, 1955). In Europe the virus is primarily transmitted by the rabbit flea (*Spilosyllus cuniculi*), whereas in Australia both the rabbit flea and mosquitoes are responsible for transmission of the virus. Both types of insects act as 'flying syringes', with no replication of the virus in the insects.

As the number of field isolates accumulated, they were graded according to their virulence, thus; Grade I (highly virulent) to Grade V (low virulence). The strains present in the UK by the 1970's were primarily Grade II or Grade IIIA (Ross, 1982). By this time the disease was estimated to be infecting about 25-27% of rabbits of which approximately 47-69% were fatally infected. In contrast, the concurrent appearance of genetic resistance within rabbit populations has led to the selection of more virulent strains of MYXV and thus a balance between virus virulence and rabbit genetic resistance appears to have been established (Ross, 1982).

Following infection, immunity to MYXV in surviving rabbits is detectable within 7 days (Fenner & Woodroofe, 1953), reaching peak levels by 28 days in survivors. Death occurs 9-13 days post infection if the virus is highly virulent, with increasing survival times for the less virulent strains. It is believed that survivors may be protected for life, even in the presence of challenge later on in life. The presence of maternal antibodies has been shown to protect young kittens for up to eight weeks (Fenner & Marshall, 1954), before they become susceptible to the virus. Typically, young kittens with no antibodies to MYXV are not naturally resistant to the virus disease.

Prior to the appearance of RHDV as an epidemic virus, seasonality was a major feature of myxomatosis (Ross *et al.*, 1989), but although periodic epidemics were often observed in the autumn (August-January) and spring (February-April)

(Vaughan & Vaughan, 1968), some evidence of the disease could be observed during all months of the year. It was generally believed that the autumn peak coincided with the loss of maternal antibodies in the recently emerged young rabbits, thus increasing the proportion of susceptible rabbits in the population. This would result in a high level of immunity in the surviving population throughout the winter. However in the spring as the vector fleas (*Spilopsyllus cuniculi*) re-emerged, the few remaining susceptible rabbits could become infected.

9.2.2 Rabbit haemorrhagic disease virus

Epidemics due to RHDV were first observed in wild rabbit populations in the UK in 1994 (Chasey, 1997). These epidemics then appeared to spread rapidly across England and Wales, although the epidemicity was always patchy with nearby rabbit populations differentially affected.

In an acute infection, the virus causes severe fulminating hepatitis and rabbits typically die within 32-76 hours post infection. The sudden onset of disease and the fact that 80% of rabbits die in the burrows mean that unless there is a really severe outbreak it is possible for the disease to pass unnoticed.

Retrospective studies on the antibody status of 70 wild rabbit populations in the UK shows that many rabbits had antibodies to RHDV prior to 1994 when the disease was first recorded (Chasey *et al.*, 1997a). The discovery of the presence of RHDV-specific antibodies in rabbits was invoked as an explanation for why some rabbits survived the initial outbreaks and why the epidemicity of the outbreaks was patchy. Furthermore, viral RNA extracted from archival domestic rabbit sera stored since the 1950's, was shown to consist of RHDV-specific sequences virtually identical with virus obtained from dead rabbits in the late 1990's (Moss *et al.*, 2002).

These data imply that an avirulent form of RHDV was circulating within the rabbit populations prior to 1994. However, from 1994 onwards epidemics in the UK due to RHDV were consistently observed and reported.

A recent study on three defined rabbit populations in the UK (White *et al.*, 2004) investigated the prevalence of RHDV-specific antibodies in rabbits over a period of 29 months. This study demonstrated an RHDV-specific seroprevalence of (80-100%) and in many individuals either increasing antibody titres over the period of observation or a sustained high antibody titre, in both cases suggesting that the animals were constantly being re-exposed to RHDV during the course of their lives.

The study reported here re-visits these sera and extends the investigation to determine, a) the prevalence of MYXV-specific antibodies in the three rabbit populations that are known to have been exposed to RHDV, and b) to see whether or not the presence of antibodies to MYXV can differentially affect the production of antibodies to RHDV, and *vice versa*.

9.3 Materials and Methods

9.3.1 Data Collection

The rabbit sera have been described previously (White *et al.*, 2004). They were collected from three sites, Exminster (Devon, OS grid reference, SX 942868), Frensham (Surrey, SU 847417), and Logiealmond (Perthshire, NN910360). Rabbits were trapped using box 'smeuse' and cage traps. Trapping was performed at 6-week intervals and lasted for 3-5 days (2-4 nights) from March 1999 to July 2001. All traps were checked every 2-3 hours during the daytime, from dawn to dusk. Rabbits caught more than once in a single trapping session were released immediately.

All rabbits were weighed, examined and tagged. A small blood sample (up to ~ 1ml) was taken from each rabbit from the marginal ear vein by shaving the region of the ear and cleaning it using a 'wet wipe' before making a small incision using a sterile blood lancet (BDH Ltd, Poole, Dorset, UK). Blood was collected in a 1.6ml microcentrifuge tube and the flow was then staunched. To prevent infection, Savlon antiseptic cream was applied to the ear after blood-sampling. Rabbits were released promptly at the site of their capture, after checking to make sure they were in an acceptable condition. The procedures performed in this study were covered by Home Office licences.

Blood samples were kept cool until delivery to the laboratory, where they were clarified by centrifugation at 2000 x g for 10 min. The serum was removed from the blood cells and stored at -20°C prior to analysis.

9.3.2 Production of Purified Myxoma Antigen

Myxoma antigen was produced as described by Gelfi *et al* (1999). Briefly, monolayers of RK13 cells were infected with a known concentration of MYXV Lausanne strain. Cells (3×10^7 per flask) infected with 1ml of virus (titre 3×10^6 pfu/ml) for 3 days and showing cytopathic effect were scraped from the flasks using a disposable silicone scraper (Falcon), sedimented by low-speed centrifugation and washed once in TL20 buffer (Gelfi *et al.*, 1999). The sedimented pellet of cells was resuspended in TL20 buffer and placed overnight at -70°C. The suspension was homogenized using a Dounce homogenizer and clarified by centrifugation at 1,200 x *g* for 10mins.

The supernatant fluid was collected and layered onto a 36% (w/v) sucrose/TL20 cushion and the virus antigen was sedimented by centrifugation at

200,000 x *g* (30,000 rpm, SW41.14 rotor) for 2 hours. The resulting virus pellet was resuspended in TL20 buffer.

9.3.3 ELISA to test for MYXV-specific antibodies

Microtitre assay plates (Dynex technologies) were coated with viral proteins (800ng to 1μg per well) diluted in PBS (pH7.6) (Gelfi *et al.*, 1999). The ELISA protocol was then followed as in Moss *et al.* (2002). The absorptions were recorded in an automated ELISA reader at a wavelength of 492 nm. Positive antibody titres were recorded as the reciprocal of the highest dilution with an absorbance 0.1 greater than the negative control serum. Sera with a titre of at least 1:20 were considered positive.

9.3.4 Seroprevalence of antibodies

Seroprevalence was estimated from the population data at each site, Exminster, Frensham and Logiealmond. For each site the number of rabbits known to be alive was estimated as described previously (White *et al.*, 2004). At the population level, seroprevalence data were analysed by applying the concept of the Minimum number of rabbits known to be alive (MNKTBA). For each site, individuals whose serological status was positive at capture and again at recapture were assumed to have a positive serological status during the period between those captures. Individuals that remained negative from one capture to another were counted as negative for the period in between. Rabbits whose serological status changed between capture points were counted as unknown for the period between those captures, rabbits who had missing data were also counted as unknown for the period between capture points. Thus for each trapping occasion the minimum

numbers known to be seropositive (MNKTBSp), seronegative, (MNKTBSn), and of unknown status (MNKTBu) were estimated. The sum of these equals the minimum number known to be alive (MNKTBA). Maximum and minimum estimates of seroprevalence were estimated using the formula:

lower estimate = MNKTBSp/MNKTBA

upper estimate = (MNKTBSp + MNKTBu)/MNKTBA.

9.3.5 Modelling interactions between RHDV and MYXV

Modelling interactions between RHDV antibodies and MYXV antibodies was performed using Generalised Linear Models with the R statistical package (www.r-project.org) (Crawley, 2005, McCulloch & Nelder, 1989). The RHDV titres were transformed to a zero to eight scale corresponding to the dilution factor used. A quasibinomial error distribution was used as some previous work indicated overdispersion. The Myxoma antigen data were counted as presence (titre > 1:10) and absence (titre ≤ 1:10). A binomial error distribution was used. Rabbits were subdivided into two categories by weight which is a suitable approximation to their age, thus, rabbits weighing < 1250g (approximately under 6 months old), and rabbits weighing > 1250g (White *et al.*, 2004). This subdivision was selected because preliminary results suggested that the interaction between RHDV and MYXV changed significantly at this point. When they reach approximately 1250g, rabbits become sexually mature. In order to exclude the possibility of pseudoreplication only one sample from each rabbit was incorporated into each dataset. For young rabbits the first datapoint for each rabbit was used. For the adult rabbits, this was either the first or last time of capture, for those animals only captured once, this datapoint was included in both datasets. In total six models were produced, three

treating RHDV antibodies as the dependent variable, with three datasets (rabbits < 1250g (n =631), rabbits > 1250g first capture (n=773), and rabbits > 1250g, last capture (n=773)), and three treating MYXV antibodies as the dependent variable with the three datasets as above.

Each model initially included all the variables Mass (continuous variable), Sex (Male/Female), Time of Year (SpringSummer/AutumnWinter), Site (Exminster/Frensham/Logiealmond), Year of Capture (1999/2000/2001) and MYXV antibodies or RHDV antibodies (factors) as single terms. Interactions between these terms were included up to the third order. The significance of individual terms was determined using deletion testing. Thus, terms were excluded from the model if the removal of the term did not significantly reduce the deviance explained by the model (Crawley, 2005). The F statistic was used to provide a test of significance in models of RHDV, which assumed a quasibinomial error distribution, and the chi-square statistic was used for the model of MYXV, which assumed a binomial error distribution. Response variables were then calculated for each model.

9.4 Results

The age and sex distribution and percentage of the population tagged are discussed at length in White *et al.* (2004), the relevant information can be found in Table 9.1.

Site	Exminster	Frensham	Logiealmond
Period of trapping	March 1999 – July 2001	March 1999 – July 2001	May 1999 – February 2001
No. rabbits tagged (female, male)	256 (119, 137)	164 (77, 87)	390 (216, 174)
No. tagged rabbits recaptured (female, male)	98 (48, 50)	62 (30, 32)	87 (47, 40)
Proportion of tagged rabbits recaptured * (female, male)	38% (40%, 36%)	38% (39%, 37%)	22% (22%, 23%)
No. capture events	578	319	495
No. blood samples†	552	277	491 (trapped) 72 (shot)
RHDV Seroprevalence (first captures): Range: Mean ‡	59% – 100% 74% (175/236)	73% – 100% § 87% (109/126)	85% – 100% 95% (367/386)
MYXV Seroprevalence (first captures): Range Mean ‡	0%-80% 55% (115/219)	50%-100% 81% (122/164)	46%-100% 89% (340/388)
Tagged cadavers found	3	2	8

Table 9.1 (From White et al. 2004)
Summary of rabbit trapping results at the three study sites
* Note that these figures include those caught on the very last trapping session that had no opportunity to be recaptured: 21 at Exminster, 30 at Frensham and 6 at Logiealmond,
† Samples suitable for analysis were not obtained at every capture event
‡ The denominators are less than the total number of rabbits that were tagged because samples suitable for analysis were not obtained from every rabbit upon first capture
§ Excluding the anomalous result of 33% in January 2000 when only three individuals were first captured

At Exminster and Frensham it was assumed that most of the population had been tagged, with approximately 90% of each month's caught rabbits having been previously tagged (except during the breeding season). Logiealmond showed a significantly lower proportion of rabbits caught that had previously been tagged.

There were no significant differences in age prevalence between the three sites (White *et al.*, 2004).

9.4.1 Immune response to Myxomatosis

We have previously observed (White *et al.*, 2004) consistently high titres of RHDV antibodies at all three sites, suggesting that the animals are constantly being re-exposed to RHDV and therefore at all three sites it is assumed that the virus is present throughout the year. It is important to note that in studying antibody titres only survivors are observed, i.e. the deaths from RHD and Myxomatosis are not counted in this study.

Seroprevalence to MYXV was high at Frensham and Logiealmond (81% and 89% respectively) for first-captured rabbits. However, at Exminster, seroprevalence to MYXV at first capture decreased markedly after June 2000, with seroprevalence being high until June 2000, March 1999 – June 2000 69.5% (105/164); and July 2000 – July 2001 34.2% (10/55).

The serological status of all recaptured rabbits is shown in Table 9.2. Almost all individuals captured at Logiealmond and Frensham that were seropositive upon first capture were always found to be seropositive when recaptured. This applied to those that were seropositive when first captured and those that seroconverted (negative to positive) after first capture. This is similar to the result seen for RHDV (White *et al.*, 2004). At these sites, it appears that immunity to MYXV is lifelong and the virus is constantly circulating thus maintaining the high levels of immunity within the population. Only one rabbit converted from seropositive to seronegative, but this could be the result of loss of maternal antibodies in the absence of exposure to the virus. However, this was the exception rather than the norm. In most cases, it

was impossible to distinguish between maternal antibodies and immune response due to virus exposure.

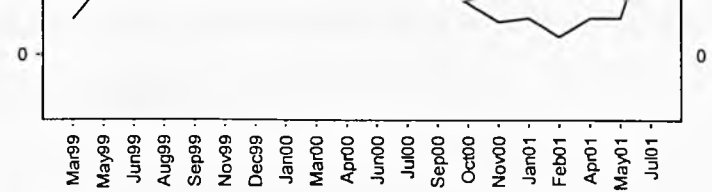
Myxoma virus Immune Status	Exminster	Frensham	Logiealmond
Positive throughout	44% (43/98)	82% (47/57)	91% (67/74)
Negative throughout	9% (9/98)	0	0
Seroconversion pos → neg	10% (10/98)	2% (1/57)	0
Seroconversion neg → pos	7% (7/98)	7% (4/57)	0
Varying	16% (15/98)	2% (1/57)	0
Unknown	14% (14/98)	7% (4/57)	9% (7/74)

Table 9.2
Showing the immune status of recaptured rabbits against Myxoma virus.
Varying means the animal showed more than one change in serological status

At Exminster, 81% (43/50) of those positive upon first capture were found to be positive upon recapture. The remaining 19% (7/50) of those found to be positive at first capture showed a gradual decrease in titre until the animals presented as seronegatives. A significant proportion of animals (16%) showed more than one change in serological status. Exminster was significantly more variable than Frensham ($\chi^2 = 73.949$, df = 18, $p < 0.001$), and Logiealmond ($\chi^2 = 45.617$, df = 15, $p < 0.001$). However, the difference between Frensham and Logiealmond was not significant.

Seroprevalence was consistently high in the study sites Frensham and Logiealmond (Fig. 9.1,b,c). Seroprevalence at Exminster (Fig. 9.1a) was consistently high until June 2000 when it decreased markedly from 89.2% to 49.7% over the course of a year (the mid-point seroprevalence estimates of Fig. 9.1a). Seroprevalence remained low at Exminster and there was a marked increase in the number of first captured rabbits presenting as seronegatives; 32/164 (March 1999-June 2000) compared to 40/55 (July 2000 – July 2001). During this latter time period, recaptured rabbits presenting as seropositives showed a decrease in titre, eventually presenting as seronegatives, with some showing subsequent conversion back to seropositives. It is interesting to note that that during the period May 2000 to July 2000 the seroprevalence to RHDV increased significantly (White *et al.*, 2004) in contrast to the MYXV seroprevalence.

Figure 9.1 (next page)
Population size (primary axis) and seroprevalence of Myxoma antibodies (secondary axis) estimates for (*a*) Exminster, (*b*) Frensham and (*c*) Logiealmond. Upper and lower seroprevalence estimates were calculated using the concept of minimum numbers known to be seropositive, seronegative and of unknown serological status on each trapping occasion (see Methods for more details). Population sizes were the minimum numbers known to be alive. Note that population sizes towards the end of the study are under estimated by this method. —, Population size; ····, seroprevalence.



(*c*) Logiealmond

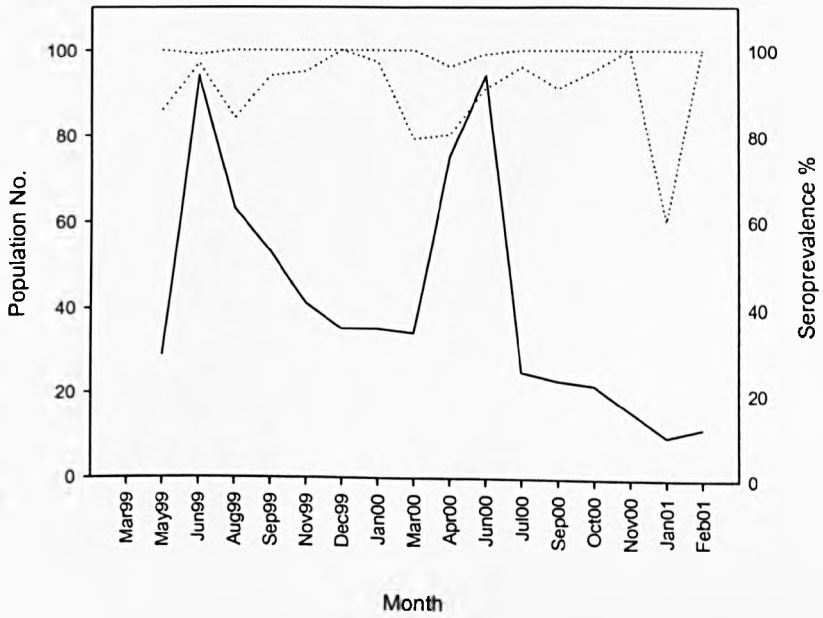
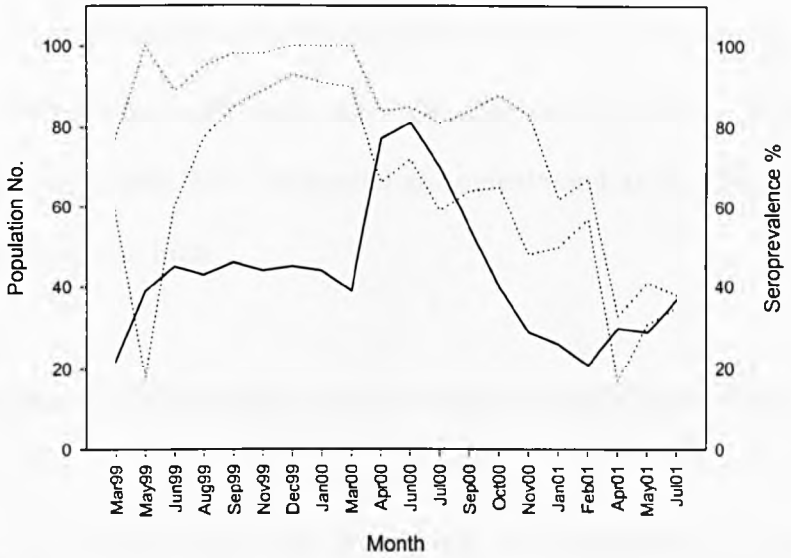
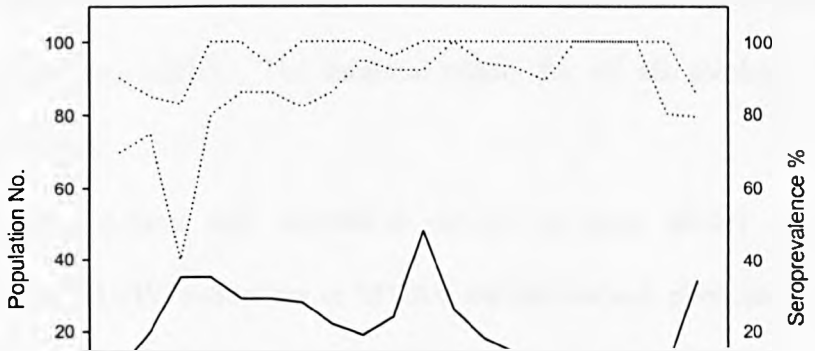


Figure 9.1

(*a*) Exminster



(*b*) Frensham



Interestingly the population size varied far more at Logiealmond (Fig. 9.1c). This is probably due to the harsher climatic conditions found in Scotland as well as the population being more widespread and seasonally more mobile. This might also explain why few rabbits were recaptured at Logiealmond as the turnover within the rabbit population is so high.

9.4.2 Determination of interdependence between MYXV antibodies and RHDV antibodies

Six Generalised Linear Models (GLMs) were produced in order to test the null hypothesis that RHDV antibodies and MYXV antibodies circulate independently within the European rabbit. The minimal model for all six models is shown in Tables 9.3 and 9.4.

For each dataset and dependent variable a basic model was produced including either RHDV antibodies or MYXV antibodies and environmental factors. Non-significant terms were removed from the model in a stepwise manner until a minimal model was produced.

RHDV antibodies as the dependent variable

(*a*)

Rabbits< 1250g (n = 631)

Term	Coefficient ± SE	F Statistic	*P*-value
Constant	-1.1712 ± 0.5434		
Mass (g)	0.0003 ± 0.0005	7.364	<0.001
MYXV antibodies	0.1339 ± 0.0388	4.917	0.002
Site Fre*	-0.8204 ± 0.7409		
Site Log	-0.600 ± 0.6708	4.636	<0.001
Year 2000*	1.0438 ± 0.3555		
Year 2001	1.2177 ± 0.4040	4.639	<0.001
Mass x Site Fre	0.0020 ± 0.0009		
Mass x Site Log	0.0019 ± 0.0008	5.911	<0.001
Site Fre x Year 2000	-0.6239 ± 0.5424		
Site Log x Year 2000	-1.8154 ± 0.4879		
Site Fre x Year 2001	-0.7929 ± 0.5641	5.004	0.002

(*b*)

Rabbits > 1250g first capture (n = 773)

Term	Coefficient ± SE	F Statistic	*P*-value
Constant	-3.6722 ± 3.6001		
Mass (g)	0.0017 ± 0.0023	2.396	NS
Sex (Female) †	2.0619 ± 2.3742	2.664	0.03
Site Fre	1.6751 ± 0.4559		
Site Log	2.0493 ± 0.4161	15.053	<0.001
Time of Year §	1.8740 ± 0.7567	2.794	0.04
Mass x Sex	-0.0007 ± 0.0.0015	3.188	0.02

(*c*)

Rabbits > 1250g last capture (n = 773)

Term	Coefficient ± SE	F Statistic	*P*-value
Constant	-3.1638 ± 3.6607		
Mass (g)	0.0014 ± 0.0023	1.092	NS
Sex (Female)	2.5417 ± 2.4079	2.655	NS
MYXV antibodies	0.0859 ± 0.0636	6.512	0.01
Site Fre	1.6829 ± 0.4573		
Site Log	2.0033 ± 0.4180	5.464	0.005
Time of Year	-0.8492 ± 2.4636	2.081	NS
Mass x Sex	-0.0010 ± 0.0015	0.033	NS
Mass x Site Fre	0.0030 ± 0.0024		
Mass x Site Log	0.0027 ± 0.0017	3.266	0.04
Mass x Time of Year	-0.0003± 0.0017	3.064	0.05
Mass x Sex x Time of Year ‡	-0.0010 ± 0.0003	5.152	0.02

Table 9.3
Effect of MYXV antibodies and other environmental factors on the titre of RHDV antibodies in wild rabbits in England and Scotland (see below for details)

MYXV antibodies as the dependent variable

(*a*)

Rabbits < 1250gm, n = 631

Term	Coefficient ± SE	□2	*P*-value
Constant	-6.829 ± 0.6713		
Mass (g)	0.0019 ± 0.0007	6.95	0.008
Site Fre*	0.0300 ± 0.5920		
Site Log	3.9938 ± 1.0947	87.36	<0.001
Year 2000*	0.2778 ± 0.5178		
Year 2001	-4.3149 ± 1.1181	32.16	<0.001
Site Fre x Year 2000	2.9092 ± 1.2048		
Site Log x Year 2000	-1.3967 ± 1.5207		
Site Fre x Year 2001	5.7685 ± 1.3112	33.90	<0.001

(*b*)

Rabbits > 1250gm first capture, n = 773

Term	Coefficient ± SE	□2	*P*-value
Constant	-5.1308 ± 2.6160		
Mass (g)	0.0056 ± 0.0018	12.33	<0.001
Year 2000	-0.5291 ± 0.6106		
Year 2001	-2.3031± 0.6441	13.60	0.001

(*c*)

Rabbits > 1250gm last capture, n = 773

Term	Coefficient ± SE	□2	*P*-value
Constant	$-8.062e^{00} \pm 3.488e^{00}$		
Mass (g)	$6.993e^{-03} \pm 2.278e^{-03}$	153.1	0.002
RHDV antibodies	$1.832e^{-01} \pm 9.931e^{-02}$	147.3	0.016
Site Fre	$2.294e^{00} \pm 1.115e^{00}$		
Site Log	$1.889e^{01} \pm 1.272e^{03}$	98.82	<0.001
Year 2000	$-2.199e^{00} \pm 1.203e^{00}$		
Year 2001	$-3.467e^{00} \pm 1.207e^{00}$	83.17	<0.001

Table 9.4
Effect of RHDV antibodies and other environmental factors on the presence or absence of MYXV antibodies in three populations of wild rabbits
* Sites Frensham and Logiealmond, and Years 2000 and 2001 were tested against Exminster and 1999 respectively.
† Sex Female was tested against Sex Male
§ Time of Year was classed as capture in Autumn/Winter and Spring/Summer, in the models Autumn/Winter was tested against Spring/Summer
‡ Where models contain interaction terms, indicative significance levels are given for component single order terms by deletion testing using models from which interaction terms had been removed

9.4.2.1 RHDV antibodies as the dependent variable

In the dataset corresponding to < 1250g, the rabbits with MYXV antibodies were statistically more likely to have RHDV antibodies ($p = 0.002$). However, for rabbits > 1250g the presence or absence of MYXV antibodies did not indicate a greater likelihood of RHDV antibodies.

The models used to predict the titre of RHDV antibodies (Table 9.3) have more terms than those used to predict the presence or absence of MYXV antibodies (Table 9.4). It is likely that this is the result of MYXV antibodies being classed as present or absent, whilst for RHDV antibodies the model is trying to predict the titre of the antibody response. This makes the model more complicated. Indeed, as titre would be expected to vary depending on the level of exposure to the circulating virus, which is in turn dependent on the climate, and other environmental factors, then the model is likely to reflect this.

For animals with weights over 1250g their antibody titres become entirely dependent on the environmental factors and the presence or absence of MYXV-specific antibodies has little or no effect on the RHDV-specific antibody levels. The two models for rabbits > 1250g are very similar with only one difference, for the rabbits at first capture the interaction term Mass x Site is significant, whilst for the last capture the interaction term Mass x Sex is significant.

9.4.2.2 MYXV antibodies as the dependent variable

For MYXV antibodies as the dependent variable the prediction of the presence or absence of MYXV antibodies within the rabbit is limited to 4 variables, Mass, Site, Year and RHDV. Sex and time of year are completely excluded from the

models. There are few interaction terms and the models are much simpler than those for RHDV antibodies.

For rabbits < 1250g the presence of RHDV antibodies does not predict the presence or absence of MYXV antibodies. Mass, Site and Year are the only significant factors required for predicting the presence or absence of MYXV antibodies. For rabbits > 1250g first capture, the model has the fewest terms, with Mass and Year of capture being most significant. Interestingly there is no difference between the sites, suggesting that the differences at the sites are determined by only the young or old rabbits not the middle age group category. The model for rabbits > 1250g last capture includes RHDV antibodies. However, the term is not highly significant and may be an artefact of the data. A more sophisticated model would be needed in order to determine how strongly the presence of RHDV antibodies influences MYXV antibodies at this point. Again the other factors are Site and Year as in the other two models.

9.5 Discussion

During the past 15 years, the focus in rabbit virology research has been on Rabbit haemorrhagic disease, rather than on Myxomatosis. Most studies in Australia have reported that the presence of RHDV circulating within the rabbit population has moved the time of Myxomatosis outbreaks to later in the year (Mutze *et al.*, 2002). It is known that the immunosuppressive nature of MYXV can cause an increased parasite burden in the rabbit (Boag, 1985). However, very little work has been done to study whether or not there are any significant interactions between RHDV and MYXV where they overlap in the wild. A recent study by Marchandeau et al (2004) estimated the relationship between RHDV- and MYXV- specific antibodies in young

rabbits. It was shown that rabbits were more likely to be antibody positive for both MYXV and RHDV than for only one of these viruses, and that both viruses were circulating concurrently. The study also demonstrated that both viruses were present in the population in the late breeding season. However, because this study was carried out only in young rabbits the results may not provide the complete picture.

9.5.1 Immune response to Myxoma virus

The capture and recapture of rabbits over a period of 28 months at three distant sites provided the opportunity for a detailed serological analysis of the relatively long-term presence of MYXV in UK rabbits. The results show that most rabbits with high titre MYXV-specific antibodies retain a high level for up to 28 months. At Frensham and Logiealmond, 86.5% of the recaptured animals show consistent high antibody titres for MYXV which is similar to the results reported for RHDV (92.7%) previously (White *et al.*, 2004) confirming that RHDV and MYXV are circulating concurrently within the populations. Thus since at these sites most rabbits are immune to MYXV the presence of RHDV does not appear to impact on exposure to MYXV. Indeed it is likely that young rabbits are exposed to MYXV whilst they are under the protection of maternal antibodies. Thus there are no obvious differences between those protected by maternal antibodies and those protected through the development of specific immunity. This is in contrast to the work done by Mutze *et al.* (2002) who found that RHDV impacted significantly on the timing of Myxomatosis outbreaks in Australia. It must be stated again that this UK study is looking at survivors rather than outbreaks. However, the immunosuppressive effects of MYXV were expected to make the animals more susceptible to RHDV. This has not been observed in this study.

Exminster shows a different pattern compared with Frensham and Logiealmond. At the time period when a significant increase in the seroprevalence of RHDV is seen, there is a corresponding decrease in the seroprevalence of MYXV (Fig. 9.2). During the period of observation, the number of first captured rabbits with MYXV-specific antibodies decreased so extensively that by April 2001 there were few new capture rabbits with MXYV-specific antibodies. The only noticeable difference between Exminster and the other sites is that during July 2000 many rabbits at Exminster died with symptoms typical of infection by RHDV. At Frensham the number of deaths was much lower.

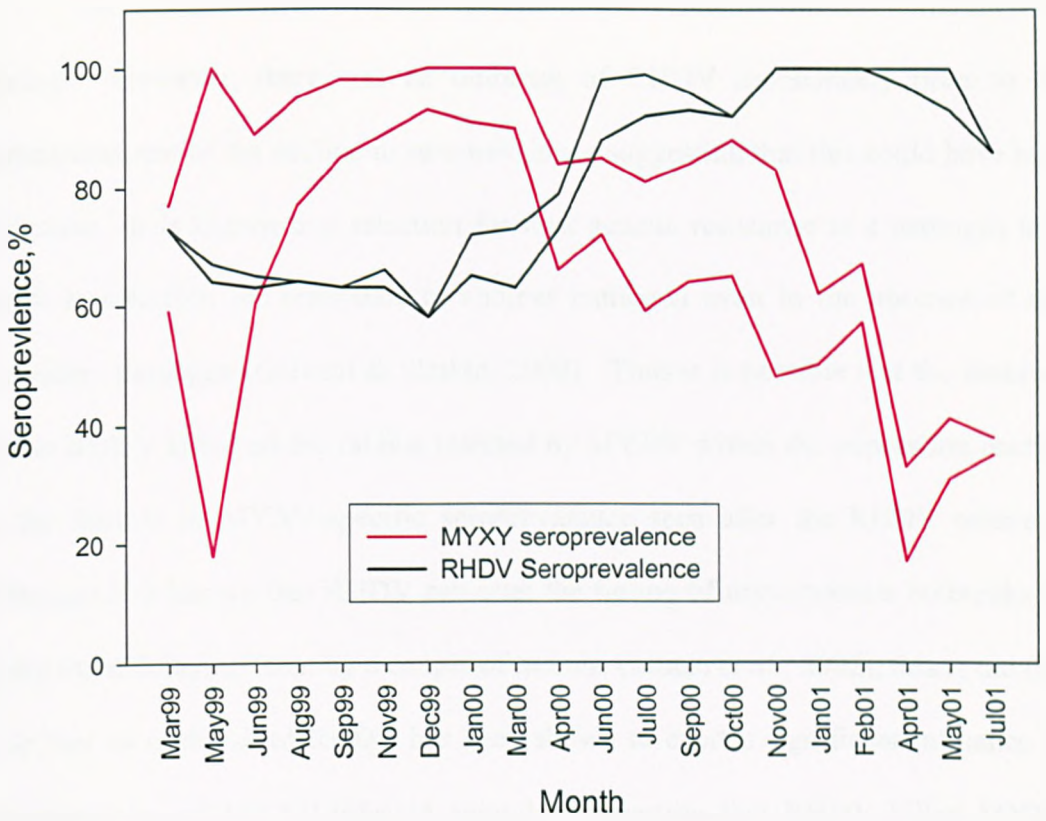


Figure 9.2
Showing the maximum and minimum levels of seroprevalence for RHDV and MYXV at Exminster between March 1999 and July 2001. Data for RHDV from White *et al.* (2004).

From August 2000 onwards rabbits previously recorded as positive for MYXV-specific antibodies showed decreasing titres, some even becoming negative, suggesting that these animals were no longer being exposed to MYXV. However, during the final two months of the long-term studies the presence of antibodies in these rabbits started to increase again indicating that the MYXV was once again circulating actively. During the period July 2000 to July 2001, the number of rabbits showing symptoms of myxomatosis, or the number that died following infection decreased continuously until February 2001 and no more rabbits showed symptoms or death after this time (results not shown).

The reasons for this sudden decline in the levels of MYXV antibodies are unclear. However, there was an outbreak of RHDV immediately prior to the commencement of the decline in seroprevalence suggesting that this could have been the cause. It is known that selection for host genetic resistance to a pathogen may result in selection for resistance to another pathogen even in the absence of this secondary pathogen (Galvani & Slatkin, 2003). Thus it is possible that the outbreak due to RHDV killed all the rabbits infected by MYXV within the population leading to the decline in MYXV-specific seroprevalence seen after the RHDV outbreak. Although it is known that RHDV can alter the timing of myxomatosis outbreaks, in many cases delaying them by a couple of months (Mutze *et al.*, 2002), this is the first time that an outbreak of RHDV has been shown to exert a significant influence on the proportion of MYXV-infected animals, suggesting that RHDV killed MYXV positive animals. Indeed, the impact of RHDV was to leave the rabbit population almost completely free of MYXV. Thus although juveniles born in 2001 will have had MXYV-specific maternal antibodies, once these had declined that cohort of the population would have been immunologically naïve to MYXV. Alternatively, it is possible that the population at Exminster simply experienced a MYXV free year, as does sometimes occur.

9.5.2 Interaction between Myxomatosis and RHDV

9.5.2.1 Young rabbits (<1250g)

We predicted from the model that the presence of RHDV antibodies can be predicted by the presence of MYXV-specific antibodies as suggested by Marchandeau *et al.* (2004). However, the presence of MYXV-specific antibodies cannot be predicted by the presence of RHDV antibodies. It is possible that this is

the result of the difference in response to the viruses during the first two months after birth. With myxomatosis the animal is protected by maternal antibodies for approximately 12 weeks. However, animals born to susceptible mothers have no protective antibodies and are susceptible to the disease (Fenner & Marshall, 1954). Animals less than two months of age are naturally resistant to RHDV either in the presence or absence of maternal antibodies. Whilst it is believed that maternal antibodies do afford some additional protection against infection with RHDV, it is known that higher titre maternal antibody increases the time that the antibodies persist within the young rabbits. The specific reason for the natural resistance of rabbits to RHDV has never been defined. It is known that in all mammals during weaning the liver undergoes a physiological change resulting from the change in diet (Balistreri & Schubert, 1987), one can therefore speculate that as the liver is the primary site of replication of RHDV in young rabbits it is unable to support replication of the virus until the change in liver physiology occurs.

Thus for approximately the first two months of life the rabbit is effectively protected from RHDV. Therefore since MYXV is likely to kill seronegative young rabbits, any survivors will be immune to MYXV and thus will be alive to develop RHDV antibodies or die of the disease. These observations effectively explain the interactions between the two viruses in young rabbits.

9.5.2.3 Adult rabbits (>1250g), first capture

With first capture rabbits at a weight greater than 1250g the models predict that the specific antibody responses to MYXV or RHDV are not significantly influenced by the presence of the other virus at the same site. Other external factors such as Mass, Sex, Site etc., may exert a significant influence on the presence and

titre of antibody. Adult first capture rabbits are just six months old and are becoming sexually mature. Less than 20% of rabbits survive to six months and those that do survive have both MYXV and RHDV antibodies. The model with MYXV antibodies as the dependent variable, predicts that the only significant factors were mass of the animal and the year in which it was captured. With RHDV antibodies as the dependent variable, the model was more complicated with more external factors involved (Table 9.3). Considering that when rabbits are approaching sexual maturity it is only a small percentage of the healthiest animals that would have survived, it seems likely that all animals would have both RHDV- and MYXV-specific antibodies and thus they would be ubiquitous throughout the populations. This high prevalence within the population excludes many interactions that might otherwise have been used to predict the presence or absence of MYXV antibodies, or the titre of RHDV antibodies.

9.5.2.3 Adult rabbits (>1250g), last capture

The models predicted that although the presence of MYXV-specific antibodies does not predict the presence of RHDV antibodies, the presence or absence of MYXV-specific antibodies can be predicted by the presence of RHDV antibodies. Rabbits with RHDV antibodies are more likely to contain antibodies against MYXV. In order to exclude the possibility that the abrupt change in seroprevalence in Exminster for both RHDV antibodies and MYXV antibodies had skewed the model, we ran two models, one for Exminster and one for Logiealmond and Frensham combined. When Exminster is considered as an individual site the interaction between MYXV antibodies and RDHV antibodies disappears. When Logiealmond and Frensham are considered as a single group characterized by high

seroprevalence for both RHDV and MYXV, the presence of MYXV antibodies can be predicted by the presence of RHDV antibodies.

Currently there is no adequate explanation for these results. It is possible that a more sophisticated model would not support this prediction. In Logiealmond and Frensham all surviving rabbits would be expected to have antibodies to both RHDV and MYXV. Moreover, since most of the rabbits have consistently high antibody titres to both viruses little or no interaction between the two would be anticipated.

In summary, the results suggest that under normal circumstances MYXV and RHDV circulate concurrently within rabbit populations. The antibody response to both appears in the wild to be lifelong, and although some animals show a declining antibody titres in some cases appearing to be negative, re-exposure to the virus results in an anamnestic response implying immune memory. The consistently high titres of antibodies at Logiealmond and Frensham demonstrate that both viruses are circulating concurrently at sufficient frequency in the wider population to challenge the study populations.

Exminster was significantly different from the other two sites, experiencing a visible epidemic outbreak of RHDV during the time that the study was underway. The evidence seems to suggest that this outbreak of RHD caused the depletion of MYXV carriers within the rabbit population; it may be that this is the result of immunosuppression engendered by MYXV in these carrier rabbits. The ability of RHDV to deplete MYXV for up to a year could indicate a possible genetic basis for survival within the rabbits, on the other hand, this could be simply due to coincidence. Whilst more work needs to be done to determine if this is the case it has been shown that rabbits likely to survive RHDV are therefore more likely to

survive myxomatosis and *vice versa*. Whilst this interaction has been observed, it has not been observed that RHDV antibodies preferentially affect the production of MYXV antibodies or *vice versa*. More work is required to study the interactions between these viruses since they could have significant implications for the use of MYXV and RHDV as bio-control agents in Australia.

Chapter 10

A search for determinants of RHDV virulence using comparative alignments of genomic length sequences

10.1 Introduction

During the course of this thesis, two full-length RHDV genomes were extracted from the liver of two geographically distinct rabbits that were healthy at the time of their capture and their complete genome sequences were determined. This provided the opportunity to look for possible genetic determinants of RHDV virulence by comparison with complete genome sequences of strains of RHDV obtained from rabbits that became infected and died. The rationale for this work was based on the knowledge that the virulence of a virus may change, either after a single point mutation or following the accumulation of several mutations each of which on its own may cause only minimal changes to the phenotype (Gritsun *et al.*, 2001). It is emphasized that this work is at a very preliminary stage.

10.2 Materials and Methods

One full-length sequence obtained from an apparently healthy rabbit in New Zealand in 2002 has been published (Forrester *et al.*, 2003). The RNA for the second analysis was extracted from the liver of another healthy rabbit that was also captured in New Zealand. This rabbit was found at a distance of 13 km from the first rabbit. The genomic-length sequence of the RNA from this second sample was determined using exactly the same procedures as those used for the first New Zealand sample (Forrester *et al.*, 2003). These genomic-length sequences were aligned with 23 other sequences listed in Table 10.1 using ClustalX (Thompson *et al.*, 1997). The resulting alignments were then imported into BioEdit (Hall, 1999) and corrections to the alignment were made manually. Differences in the nucleotide and amino acid

sequence that were unique to the healthy New Zealand rabbits were recorded and will be discussed.

Sequence	Reference	Accession No.	Country of Origin
Ascot			UK
Bahrain			Bahrain
BS89	Rossi *et al* (unpublished)	X87607	Italy
China CD	Xiang *et al* (unpublished)	AY523410	China
Czech V351	Gould *et al* (1997)	U54983	Czechoslovakia
Dachswald			Germany
Eisenhuttenstadt			Germany
Erfurt			Germany
Frankfurt5			Germany
Frankfurt12			Germany
German FRG	Meyers *et al* (1991b)	NC_001543	Germany
Hagenow			Germany
Hartmannsdorf			Germany
Iowa	Neilan *et al* (unpublished)	AF258618	USA
Jena			Germany
Meiningen			Germany
Mexico	Babcock *et al* (unpublished)	AF295785	Mexico
NZ54			New Zealand
NZ61			New Zealand
Rossi			Germany
Saudi Arabia			Saudi Arabia
SD	Rasschaert *et al* (1995)	Z29514	France
Spain 89	Parra *et al* (1990)	Z24757	Spain
Triptis			Germany
Wika			Germany

Table 10.1
The full-length RHDV sequences from dead rabbits with which NZ54 and NZ61 were compared. Sequences not referenced were identified in this thesis.

10.3 Results

Two strains of RHDV isolated from the livers of healthy rabbits were sequenced and compared with the sequences of full-length gnomes obtained from virus isolated from dead rabbits. A number of mutations unique to these two viruses were identified. They are listed in Table 10.2.

Protein	Nucleotide position	Nucleotide change	Amino Acid Change
p16	137	C to T	A to V
	223	A/T to G	
	231	A to G	
	427	C to T	P to S
p23	598	C to T	
	648	A to G	
p37	1245	T to C	
(helicase)	1272	A to T	
	1530	C to T	
	1854	C to T	
	2073	G to A	
p29	2229	A to G	
	2280	A to G	
	2437	T to C	
	2532	G to A	
	2792	T to C	V to A
	2799	G to A	
	2802	G/A to T	
	2835	T to A	
	2883	G to A	
	2934	T to C	
p15	3405	C to T	
	3618	T/G to C	
p58	3843	T to C	
(RdRp)	4261	A to G	I to V
	4494	C to T	
	4563	T to A	
	4614	G/T to C	
	4617	C to T	
	4698	C to T	
	4929	G to A	
	5215	G to A	S to N
p60	5498	A to G	
Capsid protein	5663	C to T	
	5744	G to A	
	5831	C to T	
	5873	A to G	
	5915	G/A to T	
	5951	G to A	
	6026	C to T	
	6155	A to C	
	6443	T to C	
	6452	G to A	
	6653	T to C	
	6742	C to T	A/T to V
	6888	G to A	
	6905	T to C	
	6908	T to C	

Table 10.2
Showing the nucleotide and corresponding amino acid sequence changes in healthy rabbits sequences compared with sequences isolated from dead rabbits. Nucleotide and Amino acid changes are shown as the change from virulent to avirulent at that position.

The ability of any specific change to cause an alteration in the amino acid sequence was also identified. The only full-length RHDV genomes from healthy rabbits sequenced to date are those from New Zealand, and so far it has not been possible to identify which changes result in a shift of virulence, if any, and which changes, if any, are merely the result of isolated evolution. It must be stated at this point that it has not been possible to demonstrate that the full-length RNA from healthy rabbits represents infectious virus. The results are discussed below.

10.4 Discussion

At this preliminary stage 6 unique codon changes out of 48 nucleotide substitutions have been identified in comparisons of RNA from healthy rabbits carrying complete viral genomes with RNA obtained from fatally infected rabbits (Table 10.1). As far as is known this is the first time such an analysis has been performed. However, it is important to note that these 6 amino acid changes may not all impact on virus virulence or attenuation. It will only be possible to decide whether or not individually or together they influence virus virulence when direct *in vivo* virulence studies can be performed on engineered versions of each mutation represented in Table 10.2. Of the six unique amino acid changes the substitution at nucleotide position 427 with a change from a proline to serine would be expected to result in a significant alteration of the protein conformation, which in turn could influence the initiation of translation and replication of the virus. If they have any impact at all, the amino acid changes at nucleotide positions 4261 and 5215 in the RNA-dependent RNA-polymerase (RdRp) might be expected to influence the virus replication rate. However, until more complete genome sequences isolated from the RNA of healthy rabbits and from places other than New Zealand have been

determined this interpretation can only be speculative. Moreover, it will be necessary either to culture these viruses *in vitro* or to reproduce them in rabbits before the effects of these codon changes can be properly investigated.

In the years gone by it would automatically have been assumed that silent changes have no impact on virus phenotype. However, we now know that RNA secondary structure plays a role in virus replication and protein translation. Therefore, the 42 silent substitutions should not be ignored in trying to identify the genetic determinants of RHDV virulence. It is known that conservation at synonymous site is important for the viral RNA to assume an appropriate secondary structure as demonstrated for many different single-stranded positive sense RNA viruses (Belsham & Sonenburg, 1996, Goodfellow *et al.*, 2000, Joost Haasnoot *et al.*, 2002, Mason *et al.*, 2002, Pelletier & Sonenburg, 1988, Tsukiyama Kohara *et al.*, 1992, Xiang *et al.*, 1997). Recently it has been shown that viruses which establish persistent infections may have increased levels of secondary structure (Simmonds & Smith, 1999, Simmonds *et al.*, 2004). The presence of conserved secondary structure may enable the virus to persist within the cell even in the presence of innate cell defences i.e. proteolytic enzymes. It is possible that some of these synonymous changes identified in Table 10.2 are responsible for the apparent reduction of virulence in these viruses. On the other hand it is possible that none of these sites acts as a determinant of virulence. Clearly many more sequences need to be compared and many viruses need to engineered so that they can be tested *in vivo*.

Chapter 11

Discussion

During the course of this thesis several major observations have been made.

- From the work described the first conclusion is that a known virulent virus released as a bio-control agent has been isolated as viral RNA from healthy rabbits. Since it was isolated several years after the virus was released to control the animals, this observation implies that the virus has established a persistent infection.
- Subsequent phylogenetic analysis revealed that the Chinese 1984 virus occupies a lineage that is distinct from and less divergent than several European virulent isolates that occupy more divergent lineages implying that RHDV has emerged on more than one occasion.
- Amongst the samples isolated and sequenced during this work was the first example of an attenuated virus circulating in wild rabbits. This virus, found on an island off the coast of Eire has apparently circulated amongst rabbits for at least 20 years in the absence of observed disease. Whilst it has not been formally proven by direct demonstration to be infectious under laboratory conditions, it appears to be equivalent to the Italian attenuated virus designated RCV which circulates amongst domestic rabbits.
- The high number of sequences reported in this thesis has enabled the identification of recombination in the genomes of a relatively high proportion of strains of RHDV. Moreover, the results showed that recombination can occur between relatively disparate as well as closely related strains. However, at this stage recombination does not necessarily account for the emergence of RHDV as a virulent epidemic virus.
- The characterisation of two complete genomic sequences from healthy rabbits provided a unique opportunity to begin the search for putative genetic

determinants of virulence in RHDV. Thus far, 6 amino acids were identified that may have a role to play in the virulence of RHDV.

- In looking for an explanation as to how RHDV may circulate persistently, the possibility of integration of RHDV viral RNA into cellular DNA was considered. Preliminary results indicate that RHDV may indeed use DNA as an intermediate in its survival strategy.
- It had previously been suggested that the presence of MYXV within rabbit populations could influence the survival of rabbits with RHDV. So far no evidence has been presented to suggest that this is true. Instead it appears that the MYXV and RHDV circulate concurrently amongst rabbit populations. Whilst these observations shed light on some of the RHDV biology, there are still inconsistencies in the data.

This discussion chapter re-examines the observations not yet fully explained and some of the inconsistencies that the work in this thesis has highlighted to see if they can be rationalised.

11.1 The first epidemic and dispersal across the world

From previous serological and molecular studies (Moss *et al.*, 2002, Rodak *et al.*, 1990) it had become clear that RHDV did not originate spontaneously in 1984 in China when the first recognized epidemic of RHDV was recorded (Liu *et al.*, 1984). It now appears likely that the appearance of rabbit haemorrhagic disease reflects an altered relationship between pre-existing, silently circulating RHDV and the European rabbit (*Oryctolagus cuniculus*). One of the many problems that need to be resolved is what factors led to this altered relationship.

The first epidemic of the virus in China was assumed to be the result of the introduction of Angora rabbits from Germany. However, there were no reports of any disease that could be attributable to RHDV in the farm from which the rabbits originated, or in the wild populations in Germany. This provides the first dilemma. If the Angora rabbits were healthy when introduced into China, why did an epidemic outbreak suddenly occur in domestic rabbits in China? Retrospective analysis has shown that viral RNA from healthy rabbit sera collected and stored in the 1950's is almost identical to virus sequence isolated from rabbits that died in 2000 (Moss *et al.*, 2002). It has now been demonstrated that healthy rabbits can carry genomic-length viral RNA in the absence of overt disease (Forrester *et al.*, 2003, Zheng *et al.*, 2002), although the ability of this RNA to cause infection is unknown. Therefore it is feasible that the healthy Angora rabbits may have carried the virus to China in the form of RNA, but this does not explain why the virus suddenly emerged as a virulent virus in China. The most likely scenario can be partly based on the political situation at the time. The Chinese commercial rabbit population may have been isolated from surrounding countries for a sufficiently long period of time to ensure that most rabbits did not have antibodies against RHDV. Thus they were exposed to an otherwise avirulent virus, their immunological and genetic naivety left them highly susceptible to the introduced virus. An alternative possibility is that the virus was already present in the Chinese rabbit population prior to 1984 but in an avirulent form. With this possibility the reason for the sudden switch and the timing of this apparent switch to virulence remains unclear.

It was subsequently assumed that after the first recognised epidemic in China in 1984 the epidemic virus dispersed across Asia, the Middle East and Europe. However, as shown in this thesis, no viruses, as opposed to RNA sequences, isolated

in the UK wild rabbit population occupy a phylogenetic position in the Chinese lineage. Indeed many virus strains isolated in Europe occupy more divergent lineages than that of the Chinese 1984 viral strain. Thus, the original assumption that the epidemics in the UK and in many parts of Europe arose as the result of dispersal of the Chinese virus is incorrect. Moreover, RHDV must have emerged to cause epidemics at least twice and probably several more times during the last 20 years, but at this time there is no specific explanation for why this has happened.

Several possible explanations for this apparent shift to virulence will now be considered. Firstly, the ability of closely related RNA viruses to undergo recombination has been widely documented (Dahourou *et al.*, 2002, Santti *et al.*, 1999, Twiddy & Holmes, 2003, Uzcategui *et al.*, 2001, Weaver *et al.*, 1997, Worobey & Holmes, 1999). Moreover, recombination has now been shown to occur in RHDV (Chapter 7). Thus one possibility for the emergence of virulent RHDV could be that recombination occurred between at least two strains of RHDV resulting in altered virulence for rabbits. However, the phylogenetic evidence implies that RHDV has emerged as a virulent virus on several occasions and possibly in several different countries. Therefore, recombination would have had to have taken place several times. Moreover, the sequences of viral RNA that have been detected in healthy rabbits do not appear to be significantly different from those detected in RNA from rabbits that died following infection. Thus on some occasions recombination might lead to significant changes between the viruses, but on others it clearly does not. More evidence is required before the significance of recombination and RHDV virulence can be accurately assessed.

Secondly, it is possible that the change from avirulent to virulent RHDV requires only a minimal number of mutations. Such a scenario would be consistent

with the proposal that RHDV has emerged as a virulent virus on several or even many occasions. However, in many cases the scale of the outbreaks was possibly so small that they were missed or overlooked. On the other hand, the Chinese outbreak and subsequent outbreaks due to strains descended from this Chinese virus may have occurred mainly in geographic regions where rabbit immunological naivety predominated.

A third explanation for the emergence of RHDV as a virulent virus takes into account the accumulation of neutral (silent) mutations as described in the neutral theory of evolution (Kimura, 1968, King & Jukes, 1969), suggesting that these silent mutations caused the abrupt shift in phenotype. This theory makes several assumptions namely, (a) that the virus circulates as a persistent infection with a slow replication rate, just sufficiently so that RHDV survives to infect a new generation, and (b) that in this persistent state the secondary structure of the viral RNA is highly conserved. Under these circumstances one can propose a mechanism for the global emergence of RHDV as a virulent virus. During the persistent state replication is relatively slow, whilst conversely, the evolutionary constraints on the virus tend to increase (Nichol *et al.*, 1993), mainly due to the need to maintain a functional secondary structure. Thus, the need to conserve the synonymous sites will increase. In other words, mutations that severely disrupt RNA secondary structure will not produce viable virus. These evolutionary constraints on the virus will effectively reduce the rate at which the virus can accumulate mutations (Simmonds & Smith, 1999, Smith & Simmonds, 1997). However, mutations that do not cause any significant disruption to RNA secondary structure will accumulate and be fixed by random drift under the constraints of the neutral theory which assumes that these mutations will occur at a constant rate. Over a long period of time these neutral

mutations will accumulate, gradually opening up the RNA secondary structure until an additional single mutation is sufficient to ensure that the RNA replicates rapidly and makes the virus virulent. During this period of increased replication, mutations accumulate at a faster rate aided by the lack of constraints on RNA secondary structure. Over a period of time, it is possible that the virus will once again revert to an avirulent form as mutations re-establish the conserved RNA secondary structure. Although no results were presented in this thesis, preliminary analysis of the level of synonymous variation of the 25 full genome sequences generated by this thesis suggests that RHDV may have the RNA characteristics of a persistent virus (results not shown). However, the Minimum Free Energy (MFE) produced upon folding of the RNA into a secondary structure is very low, which is consistent with non-persistent viruses rather than persistent viruses (Simmonds *et al.*, 2004). With persistent viruses the secondary structure needs to be assumed quickly in order to prevent degradation. Therefore usually, the virus exhibits a high MFE enabling the secondary structure to be readily assumed. Preliminary analysis of virulent strains of RHDV showed little MFE, suggesting that adopting the correct conformation of secondary structure is unimportant. It will be interesting to see if the reverse is true when a database of viral RNA sequences has been accumulated from healthy rabbits. If these viruses exhibit a high free energy this would add weight to the hypothesis that RHDV is typically a persistent virus. If this boom and bust hypothesis is correct, then RHDV could have switched from virulent to avirulent and back again. If this does occur periodically, the accumulation of these neutral mutations could occur at a constant rate, and thus all the viruses could suddenly switch within a relatively short period of time, in this case ten years or so. It is recognised that this third possibility

is highly speculative, but it raises questions that will need to be addressed to further our understanding of the evolution of RHDV.

11.2 Antibody protection against RHDV

Strains of RHDV from different geographic regions are known to cross-react antigenically and vaccines based either on extracted viral proteins or on recombinant capsid proteins have been shown to protect rabbits from infection with RHDV (Barcena *et al.*, 2000, Bertagnoli *et al.*, 1996, Castanon *et al.*, 1999, Fischer *et al.*, 1997, Marin *et al.*, 1995, Plana Duran *et al.*, 1996, Torres *et al.*, 2001). Therefore the conventional belief is that rabbits with antibody to RHDV should be protected from the fatal effects of this virus. However, previous publications (Moss *et al.*, 2002, Nagesha *et al.*, 2000, O'Keefe *et al.*, 1999, Robinson *et al.*, 2002a, Rodak *et al.*, 1990) and results presented in this thesis have shown that prior to the first recognition of epidemic RHDV, the presence of antibodies was ubiquitous in every rabbit population tested. Moreover, antibodies are present in rabbits that succumb to infection by RHDV (Calvete *et al.*, 2002, Marchandeau *et al.*, 2005). Clearly, this is an oversimplification but these apparent contradictions need to be explained. One possibility could be that in countries or regions where epidemics arise, the virus that has been circulating prior to the epidemic is significantly different from the epidemic virus, and therefore has induced antibodies that are not highly cross-protective against the incoming epidemic virus. The strains Ashington, Lambay and RCV which show only about 80% nucleotide identity to more recently introduced strains are typical examples of viruses that might fit these situations. Whilst such viral heterogeneity clearly exists amongst the rabbit populations it is still not this simple because it has been demonstrated that RCV protects animals from infection with RHDV (Capucci *et al.*, 1996b). However, such experimentally induced cross-

protective antibodies may not represent the true field situation in which the antibody titres may be quite low so that these animals cannot respond sufficiently rapidly to a highly virulent and antigenically different virus and are thus effectively non-immune.

Whilst this possibility cannot be ruled out, other factors must also be involved because there is evidence that rabbits with very high antibody titres still die following infection with RHDV (> 1:1280) (R.Trout – personal communication).

An additional possibility takes into account the conformation of the viral capsid protein. The 90 dimers of the capsid (C) protein (Martinez-Torrecuadrada *et al.*, 1998) form a shell domain with arch-like capsomers (Fig 11.1). The N-terminal region of the C protein forms the shell and the C-terminal region forms the arches (Granzow *et al.*, 1996, Laurent *et al.*, 2002, Laurent *et al.*, 1997). The N-terminal region is therefore buried beneath the arches and is less readily accessible to the antibodies of the host animal. Indeed it has been shown that only 50% of antibody sites can be occupied on a single virion due to steric interference (Thouvenin *et al.*, 1997). This steric hindrance may in some way enable the virus to escape from the immune system.

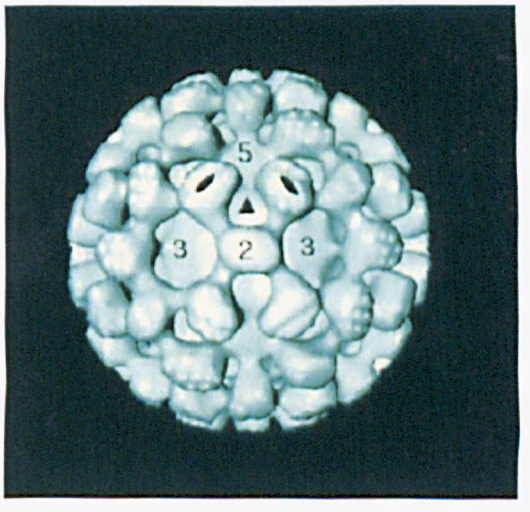


Figure 11.1
Showing the conformation of the capsid protein when virion structure is assumed. Numbers indicate number of axes present, figure from Thouvenin *et al.* (1997)

It is also known that if a rabbit experiences a sub-lethal infection then a new type of RHDV is produced called smooth RHDV (sRHDV) or core-like particles (CLP's) (Granzow *et al.*, 1996, Schirrmeier *et al.*, 1997). It can be argued that if the antibodies bind preferentially to the N-terminal region of the C protein as suggested by Martinez-Torrecuada *et al.* (1998) then the production of sRHDV would render the antibodies more able to clear the virus from the system. Thus, the survival of the animal could depend on the proportion of sRHDV to wild type (wt) RHDV present in the rabbit. If the animal is infected mainly with wt RHDV then the infection is more likely to overwhelm the animal as the antibody binding would be poor, whereas if a higher proportion of virus comprises sRHDV antibody binding would be more efficient and the animal would be less likely to be overwhelmed. This could explain why the amount of infectious virus appears not to be important in determining mortality (Teifke *et al.*, 2002). Instead it could be argued that it is the proportion of wt RHDV that determines the survival of the animal.

Whilst this concept might explain how immunity fails to protect against infection, it does not explain why a sub-lethal infection leads progressively to an increase in the proportion of sRHDV. One can speculate that sRHDV represents an incomplete form of RHDV that can pass from cell to cell without being released from the cells in large quantities. Thus in the presence of antibodies, sRHDV would effectively have an advantage and become the predominant population. It has been suggested that the production of sRHDV is due to truncated gene expression (Barbieri *et al.*, 1997). This would be consistent with the notion of incomplete virions suggested above. Whatever the nature of sRHDV compared with wtRHDV it is likely that this is an integral part of the antibody escape mechanism of the virus.

Another important, but not yet properly assessed possibility that could impact on RHDV virulence is immune mimicry. It is known that viral and cellular epitopes may share antigenic properties and this form of mimicry could have significance in viral immunopathogenesis (Barnett & Fujinami, 1992, Falconar, 1997, Gould *et al.*, 1983, Oldstone, 1987). Antibody mediated immunopathology has not been associated with disease due to RHDV. However, the rapidity with which infection by RHDV progresses is reminiscent of the observations that Yellow Fever virus and Japanese Encephalitis virus virulence for mice can be significantly increased in the presence of monoclonal antibodies that bind viral, and in some cases cellular epitopes (Gould & Buckley, 1989). Thus there is a case for considering whether or not antigenic mimicry could explain the rapid onset of death due to RHDV. Alternatively, antibody mediated disease enhancement is well known for dengue viruses. In this case it has been shown that secondary dengue virus infections of humans by dengue virus serotypes, different from that causing the primary infection, often lead to the development of haemorrhagic fever or shock syndrome (Halstead, 2003). The mechanism is believed to be primarily due to failure of the heterologous antibody to neutralise the infectivity of the heterologous virus. Fc-receptors on immune cells such as macrophages bind to these non-neutralising antibodies which are complexed with virus. These complexes effectively target the virus to the immune cells and trigger immunopathological reactions that ultimately progress to severe and often fatal haemorrhagic disease (Halstead & Simasthien, 1970). Perhaps antibody-mediated enhancement of RHDV virus infectivity could explain the rapid onset of disease.

11.3 Evolution of the virus

Direct evidence presented in this thesis and in other publications (Moss *et al.*, 2002, Rodak *et al.*, 1990) has demonstrated directly that RHDV has circulated apparently harmlessly in rabbits for the past fifty years. Moreover, the phylogenetic evidence infers that this virus has circulated harmlessly or as a virulent virus for many centuries or perhaps even millennia. Nevertheless, the evolutionary origins of RHDV have not been considered beyond a period of fifty years from the present time.

RHDV is a member of the *Calciviridae* family in the genus *Lagovirus*, which currently includes RHDV and EBHSV (Fauquet *et al.*, 2004). Among the Lagomorphs, only the European rabbit and the European Brown Hare have been shown to circulate caliciviruses within their populations, although there is anecdotal evidence of a disease similar to EBHS in the North American Snowshoe hare (*Lepus americanus*). This disease termed 'shock disease' kills hares in periodic cycles of about 10 years. Hares that die of this illness assume the characteristic posture of RHDV (see Fig. 1.1), and infected animals appear normal until just prior to death (Green *et al.*, 1939). Post-mortem examination revealed that liver and spleen were preferentially infected. If this shock disease agent is a member of the genus *Lagovirus*, then there are at least two possibilities as to its origin. Firstly, that it was introduced from Europe into the *L. americanus* population. Secondly, considering the evidence that RHDV is potentially a persistent virus it is possible that these viruses have co-evolved with the animals and that the speciation of the animal has led to genetic isolation of the viruses and the different species seen today. Thirdly, the virus could have been introduced from one of the mammalian or avian hosts that are infected with caliciviruses, this may also be true for RHDV and EBHSV.

Other genera of viruses within the family *Caliciviridae* infect a wide range of mammalian and avian hosts. For example, Vesicular Exanthema of Swine virus (VESV) infects pigs, and San Miguel Sea Lion virus can infect Sea Lions, although it is very closely related to VESV. Caliciviruses infect Cats (Feline calicivirus), and Cows (Bovine calicivirus), as well as primates (Primate calicivirus) and humans. It is therefore entirely possible that RHDV and EBHSV originally evolved from divergent lineages of viruses that simultaneously diverged to produce viruses that specifically infect humans, cats, pigs, seals, etc. Although there have been some attempts to examine the evolutionary relationships between caliciviruses (Berke *et al.*, 1997, Berke & Matson, 2000), so far no indication of the origin of RHDV or EBHSV has been obtained from these analyses.

11.4 Climate and its impact on RHDV

When RHDV is introduced for the first time into wild rabbits distinct epidemiological patterns are observed. For example, when RHDV escaped from Wardang Island onto mainland Australia, the epidemicity of the virus appeared to depend on the prevailing climate. In drier climates there were high levels of disease, the epidemic outbreaks lasted for relatively long periods of time and the affected rabbit populations remained at low levels for considerably longer periods of time than was observed in wetter climates. In contrast, in regions with significantly more rainfall, a patchy epidemicity was observed, with occasional local extinctions, but these extinctions were only at the micropopulation level, rather than within the general rabbit population of the region under study.

It is also known that climatic perturbation affects rabbit population density. In drier climates, gene flow between rabbits has been shown to span up to 1600km^2

(Fuller *et al.*, 1997) and the effective population size is much greater than is observed amongst single breeding colonies. The drier climate drives constant population turnover due to local extinction and recolonization. Conversely in wetter areas, there is less gene flow between local populations which show a high degree of non-random mating and social structuring and the effective population size is maintained at much lower levels (Surridge *et al.*, 1999a, Surridge *et al.*, 1999b).

At least two factors may drive the intensity of an RHDV epidemic. Firstly, in a large rabbit population the number of susceptible rabbits required to sustain an epidemic is reached early in the breeding season when many of the young rabbits are resistant to infection, thus more rabbits survive to become the breeding population in the following year. In smaller populations the number of susceptible animals is obviously lower causing epidemics to arise later in the season when many of the juveniles have lost their natural resistance and maternally acquired immunity. Under these circumstances the impact of the epidemic would be more pronounced, as very few rabbits would survive to the next breeding season, leading to a decline in the population. In other words, we would expect to see a lower impact following epidemics in the larger populations and a higher impact in the smaller populations.

However, in a drier climate, the home range of the rabbits is extended due to the need to forage for food and water. Thus there is the possibility of more contact between the populations leading to an increased likelihood of susceptible rabbits encountering contagious animals. Conversely in wetter areas, the abundance of food and water reduces the home range, leading to reduced contact rates between populations, and therefore less chance of exposure to RHDV. Therefore, although in the smaller populations a single epidemic may be devastating, it is likely to be an isolated event, and the affected population will either experience extinction or

recovery depending on the prevailing antibody status in the population and the time between epidemics. In larger populations in dry areas, the increased contact between populations, and other factors such as stress result in continuous transmission of the virus throughout the year although there will be more significant outbreaks during the breeding season. Thus, the virus becomes endemic under these circumstances maintaining the population at the depressed level. This hypothesis could explain the observation of deaths throughout the year in drier climates despite the absence of large epidemics (Cooke, 2002, Marcato *et al.*, 1991). In contrast a recent study in New Zealand which has a wetter climate showed the absence of migration (Henning, 2003) and therefore reduced exposure to the virus. This lack of contact between rabbit populations could also explain why high mortality outbreaks have been observed in local populations whilst others nearby remain free of disease.

Whilst the extremes of climate and population size have been discussed above in order to emphasise the differences. In reality most rabbit populations will vary significantly in size, contact rates between populations and the climate in which they find themselves. It is likely that a combination of all of these will drive the intensity and scale of the epidemic. How these theories tie in with previous work on the spread of RHDV and the structure of rabbit populations (Boots *et al.*, 2004) remains to be determined.

Another plausible explanation for the difference in the response of the rabbits to RHDV in differing climates is the effect of stress. Stress has been shown to cause immune modulation and whilst acute stress can enhance the immune system, chronic stress results in immunosuppression (Dhabhar, 2000, Irwin, 1993). In drier climates the animals are stressed because they are under pressure to find food and water, it is possible that as a consequence of this their resistance to RHDV is reduced and thus

greater mortality is found within the animals. Conversely, in wetter climates the animals are not subject to this form of stress. Interestingly, rabbits imported from the wetter areas of Spain, where RHDV has had a reduced impact, into the more arid areas where the numbers of rabbits are dangerously low, exhibit a higher level of mortality when transported to the drier area, again possibly as the result of stress.

The animals may also suffer stress resulting from commercial transportation. To date, the impact of introducing rabbits into other countries for trade and breeding purposes has not been discussed. The demand for rabbits and their produce is enormous and many rabbits are transported for new breeding stock or for food, and or fur products. It has been noted that epidemics frequently arise soon after the introduction of new breeding stock into a rabbitry. Such outbreaks are often confined to the rabbitry into which the rabbits were introduced (unpublished observation). Whilst the presence of immunologically naïve rabbits in the destination rabbitry has been discussed as a potential cause of an outbreak, one other explanation is the stress engendered in the rabbits arriving in the rabbitry due to the transportation. Such stress might cause an avirulent infection to become virulent and regardless of the immunological status of the rabbits within the rabbitry this could result in an epidemic outbreak.

Overcrowding is another source of stress. Rabbits in the wild exhibit severe boom and bust cycles (Middleton, 1934), and overcrowding is especially common in Australia and New Zealand (Gibb & Williams, 1994, Wood, 1980). Whilst it has not been demonstrated directly that overcrowding triggers disease, it was suggested that this could have been the cause of the outbreak of 'Shock disease' in the Snowshoe hare in Canada (Green *et al.*, 1939). There is also anecdotal evidence that during 1894 rabbits in England died of a disease that was similar to RHDV (Simpson,

1893), showing the same patchy epidemicity. It is also possible that the co-circulation of MYXV masks the effects of RHDV, although at the present time no interaction between RHDV and MYXV has been observed. Moreover, MYXV has never been introduced into New Zealand, and the rabbits showed high levels of overcrowding in the absence of any disease such as RHDV for the last 50 years. It must be emphasised that all these reports are anecdotal. However, the effects of stress should not be ignored as a potential mechanism by which RHDV epidemicity increases.

Another possible explanation for the differential impact of RHDV on the rabbit in different climates is that in wet climates the virus cannot survive for as long in the environment as it can in drier climates. Indeed, in dry climates, in the absence of water and protection from UV light, i.e. in a burrow, the virus is likely to survive longer than in a wetter climate, where the presence of moisture means that enzymes are more likely to be able to inactivate the virus.

11.5 Implications for RHDV as a bio-control agent

The initial impact of epidemic RHDV in China in 1984 and then in parts of Asia, the Middle East and Europe together with the need to control rabbit densities in Australia and New Zealand led to RHDV being considered as a potential agent for bio-control. Unfortunately the manner by which RHDV was initially released onto mainland Australia did not enable extensive testing on the long-term effects of RHDV on rabbit populations to be assessed. The initial escape of RHDV onto the Australian mainland from Wardang Island was believed to be accidental. However, the rapidity with which the virus dispersed and killed rabbits in Australia led to the initial conclusion that RHDV was an effective bio-control agent (Kovaliski, 1998).

The initial success of the virus as a bio-control agent is almost certainly in part, due to the fact that at the time of the release the country was experiencing very dry conditions. In addition, there were coincidentally large populations of insects present at the time of release (Lugton, 1999) and it is believed that insects may have contributed significantly, as mechanical vectors, to the spread of the virus. Nevertheless, the success of this virus as a bio-control agent has now begun to be questioned because in some parts of Australia the rabbit population densities are now reaching pre-RHD levels. New Zealand has also experienced a similar disparity in the impact of the virus, effective in some regions and less effective in others. Moreover in New Zealand the virus readily establishes avirulent infections (Forrester *et al.*, 2003, Zheng *et al.*, 2002) suggesting that the effectiveness of the virus as bio-control agent may not be sustained over long periods of time. Very recently, there have been new reports from New Zealand that the effectiveness of the virus has decreased as a bio-control agent and thus a second release is planned (B. Boag – personal communication). Considering the results and discussions presented here, what are the possible implications for RHDV as a bio-control agent?

Firstly, it has been demonstrated that almost 50% of rabbits tested in New Zealand three years after its first release carry the virus in form of viral RNA in the absence of overt disease (Forrester *et al.*, 2003). This implies that the virus is infecting a high proportion of rabbits without killing them and the presence of the viral RNA in the liver of these rabbits implies that the virus may have established persistent infections in these rabbits. Moreover, whilst several genetic changes were detected in the RNA sequences isolated from healthy rabbits, it is unknown at these times which, if any, are responsible for the apparent reduction in virulence of the bio-control agent.

Secondly, at this time and by analogy with what happened for MYXV (Ross & Sanders, 1984) it is not known if genetic resistance of the rabbits to RHDV has been selected. If this did prove to be the case, then presumably the introduction of a distinct strain of RHDV as a bio-control agent would be to no avail.

Thirdly, evidence based on phylogenetic analysis has been presented to support the idea that RHDV has been circulating in European rabbits for many years, possibly thousands, and this is reflected in the observation of the widely disparate strains of virus, viz., Ashington, Lambay and RCV compared with other extant strains isolated from diverse areas of Europe, Asia and the Middle East. Whilst there is still significant variation amongst these latter strains, they show significantly less genetic heterogeneity than Ashington, Lambay and RCV, presumably inferring that they evolved relatively recently. Nevertheless, even these lineages may represent evolution over a period of centuries.

How can we explain this apparently low rate of evolution compared with other viruses such as HIV and Hepatitis C virus? The most likely explanation comes from the observation that RHDV can apparently develop persistent infections in rabbits during which there is very little turnover of the virus. Under these circumstances whilst the rate of mutation would be identical to that of epidemic virus, the low virus turnover would constrain variation due to mutation. The preliminary discovery of a conserved rate of synonymous sites in viral RNA that is characteristic of persistent viruses demonstrates the need to retain secondary RNA structure and thus supports the idea of persistence.

It therefore seems possible that the use of RHDV as a bio-control agent could result in the development of a situation not unlike the one that appears to have been established on Lambay Island, i.e. healthy rabbits circulating a harmless virus

through many generations with no suggestion of epidemic outbreaks. Presumably, on the basis of the serological evidence presented in this thesis, the introduction of a recognized virulent strain of RHDV would have had little or no impact on the rabbits because they already have high titre antibodies to the Lambay Island strain. However, without direct evidence this can only be considered a speculative comment. One way in which this concept could be tested would be to stop completely the release of the Czech strain of RHDV in New Zealand for a period of five years and then prior to releasing it again perform wide ranging serological tests for the presence of RHDV antibody. If the RHDV epidemicity ceases or drops to low levels during the five year period one might expect to see antibody levels dropping accordingly. However, if a situation such as that seen on Lambay pertains, the antibody levels should remain high.

The work presented in this thesis has provided novel and interesting findings, many of which raise important issues that require resolution if we are to understand more fully the epidemiology of RHDV.

Bibliography

Abu Elzein, E. M. & al-Afaleq, A. I. (1999). Rabbit haemorrhagic disease in Saudi Arabia. *Veterinary Record* **144**, 480-481.

Allan, R. M. (1956). A study of the populations of the rabbit flea *Spilopsyllus cuniculi* (Dale) on the wild rabbit *Oryctolagus cuniculus* in north-east Scotland. *Proceedings of the Royal Entomological Society of London (A)* **31**, 145-152.

Alonso, C., Oviedo, J. M., Martin Alonso, J. M., Diaz, E., Boga, J. A. & Parra, F. (1998). Programmed cell death in the pathogenesis of rabbit hemorrhagic disease. *Archives of Virology* **143**, 321-332.

Altschul, S. F., Gish, W., Miller, W., Myers, E. W. & Lipman, D. J. (1990). Basic local alignment search tool. *Journal of Molecular Biology* **215**, 403-410.

Andrewes, C. H., Muirhead-Thompson, R. C. & Stevenson, J. P. (1956). Laboratory studies of *Anopheles atroparvus* in relation to myxomatosis. *Journal of Hygiene* **54**, 478-486.

Angulo, E. & Cooke, B. (2002). First synthesize new viruses then regulate their release? The case of the wild rabbit. *Molecular Ecology* **11**, 2701-2709.

Aragão, H. d. B. (1943). O virus do mixoma do coelho do mato (*Sylvilagus minensis*) sua transmissão peles *Aedes scapularis* e *aegypti*. *Memorias do Instituto Oswaldo Cruz (Rio de Janeiro)* **20**, 225-247.

Armour, C. J. & Thompson, H. V. (1955). Spread of myxomatosis in the first outbreak in Great Britain. *Annals of Applied Biology* **43**, 511-517.

Asgari, S., Hardy, J. R. & Cooke, B. D. (1999). Sequence analysis of rabbit haemorrhagic disease virus (RHDV) in Australia: alterations after its release. *Archives of Virology* **144**, 135-145.

Asgari, S., Hardy, J. R., Sinclair, R. G. & Cooke, B. D. (1998). Field evidence for mechanical transmission of rabbit haemorrhagic disease virus (RHDV) by flies (Diptera:Calliphoridae) among wild rabbits in Australia. *Virus Research* **54**, 123-132.

Balistreri, W. F. & Schubert, W. K. (1987). Liver disease in infancy and childhood. In *Diseases of the Liver*, 6th Edition edn, pp. 1337-1426. Edited by L. Schiff & E. R. Schiff. Philadelphia: J. B. Lippencott Company.

Banner, L. R. & Lai, M. M. (1991). Random nature of coronavirus RNA recombination in the absence of selection pressure. *Virology* **185**, 441-445.

Barbieri, I., Lavazza, A., Brocchi, E., Konig, M. & Capucci, L. (1997). Morphological, structural and antigenic modifications of rabbit haemorrhagic disease virus in the course of the disease. In *Proceedings of the first international symposium on Caliciviruses*, pp. 182-193. Edited by D. Chasey, R. M. Gaskell & I. N. Clarke. Reading, UK. European Society for Veterinary Virology.

Barcena, J., Morales, M., Vazquez, B., Boga, J. A., Parra, F., Lucientes, J., Pages Mante, A., Sanchez Vizcaino, J. M., Blasco, R. & Torres, J. M. (2000). Horizontal transmissible protection against myxomatosis and rabbit hemorrhagic disease by using a recombinant myxoma virus. *Journal of Virology* **74**, 1114-1123.

Barlow, N. D., Barron, M. C. & Parkes, J. P. (2002). Rabbit haemorrhagic disease in New Zealand: field test of a disease-host model. *Wildlife Research* **29**, 649-653.

Barnett, L. A. & Fujinami, R. S. (1992). Molecular mimicry: a mechanism for autoimmune injury. *FASEB Journal* **6**, 840-844.

Barratt-Hamilton, G. E. H. (1912). A history of British mammals. London: Gurney and Jackson.

Battersby, J. E. (2005). UK mammals: Species status and population trends. Peterborough: JNCC/Tracking Mammals Partnership.

Bello, G., Casado, C., Garcia, S., Rodriguez, C., del Romero, J. & Lopez-Galindez, C. (2004). Co-existence of recent and ancestral nucleotide sequences in viral quasispecies of human immunodeficiency virus type 1 patients. *Journal of General Virology* **85**, 399-407.

Belsham, G. J. & Sonenburg, N. (1996). RNA-protein interactions in regulation of Picornavirus RNA translation. *Microbiology Reviews* **60**, 499-511.

Berke, T., Golding, B., Jiang, X., Cubitt, D. W., Wolfaardt, M., Smith, A. W. & Matson, D. O. (1997). Phylogenetic analysis of the Caliciviruses. *Journal of Medical Virology* **52**, 419-424.

Berke, T. & Matson, D. O. (2000). Reclassification of the *Caliciviridae* into distinct genera and exclusion of hepatitis E virus from the family on the basis of comparative phylogenetic analysis. *Archives of Virology* **145**, 1421-1436.

Berninger, M. L. & House, C. (1995). Serologic comparison of four isolates of rabbit hemorrhagic disease virus. *Veterinary Microbiology* **47**, 157-165.

Bertagnoli, S., Gelfi, J., Le Gall, G., Boilletot, E., Vautherot, J. F., Rasschaert, D., Laurent, S., Petit, F., Boucraut Baralon, C. & Milon, A. (1996). Protection against myxomatosis and rabbit viral hemorrhagic disease with recombinant myxoma viruses expressing rabbit hemorrhagic disease virus capsid protein. *Journal of Virology* **70**, 5061-5066.

Blair-West, J. R., Coghlan, J. P., Denton, D. A., Nelson, J. F., Orchard, E., Scoggins, B. A., Wright, R. D., Myers, K. & Junqueira, C. L. (1968). Physiological, morphological and behavioural adaptation to a sodium deficient environment by wild native Australian and introduced species of animals. *Nature* **217**, 922-928.

Boag, B. (1989). Population dynamics of parasites of the wild rabbit. In *Mammals as Pests*, pp. 186-195. Edited by P. J. Putnam. London: Chapman and Hall.

Boga, J. A., Casais, R., Marin, M. S., Martin Alonso, J. M., Carmenes, R. S., Prieto, M. & Parra, F. (1994). Molecular cloning, sequencing and expression in Escherichia coli of the capsid protein gene from rabbit haemorrhagic disease virus (Spanish isolate AST/89). *Journal of General Virology* **75**, 2409-2413.

Boga, J. A., Marin, M. S., Casais, R., Prieto, M. & Parra, F. (1992). In vitro translation of a subgenomic mRNA from purified virions of the Spanish field isolate AST/89 of rabbit hemorrhagic disease virus (RHDV). *Virus Research* **26**, 33-40.

Boga, J. A., Martin Alonso, J. M., Marin, M. S., Casais, R., Vazquez, A. L., Machin, A. & Parra, F. (1999). Genome organisation and gene expression strategies of rabbit haemorrhagic disease virus. *Recent research and developments in Virology* **1**, 107-119.

Boniotti, B., Wirblich, C., Sibilia, M., Meyers, G., Thiel, H. J. & Rossi, C. (1994). Identification and characterization of a 3C-like protease from rabbit hemorrhagic disease virus, a calicivirus. *Journal of Virology* **68**, 6487-6495.

Boots, M., Hudson, P. J. & Sasaki, A. (2004). Large shifts in pathogen virulence relate to host population structure. *Science* **303**, 842-844.

Bouslama, A., De Mia, G. M., Hammami, S., Aouina, T., Soussi, H. & Frescura, T. (1996). Identification of the virus of rabbit haemorrhagic disease in Tunisia. *Veterinary Record* **138**, 108-110.

Bouvier, G. (1954). Quelques remarques sur la myxomatose. *Bulletin de l'Office International des Épizooties* **46**, 76-77.

Brambell, F. W. R. (1944). The reproduction of the wild rabbit, *Oryctolagus cuniculus*. *Proceedings of the Zoological Society, London* **114**, 1-45.

Brault, A. C., M., P. A., Holmes, E. C., Woelk, C. H. & Weaver, S. C. (2002). Positively charged amino acid substitutions in the E2 envelope glycoprotein are associated with the emergence of Venezuelan Equine Encephalitis Virus. *Journal of Virology* **76**, 1718-1730.

Brown, P. W., Allan, R. M. & Shanks, P. L. (1956). Rabbits and myxomatosis in the North-East of Scotland. *Scottish Agriculture* **35**, 204-207.

Calvete, C., Estrada, R., Lucientes, J., Osacar, J. J. & Villafuerte, R. (2004). Effects of vaccination against viral haemorrhagic disease and myxomatosis on long-term mortality rates of European wild rabbits. *Veterinary Record* **155**, 388-392.

Calvete, C., Estrada, R., Villafuerte, R., Osacar, J. J. & Lucientes, J. (1995). Primeros resultados encampo sobre la enfermedad hemorrhagica del conejo. *Trofeo* **304**, 22-28.

Calvete, C., Estrada, R., Villafuerte, R., Osacar, J. J. & Lucientes, J. (2002). Epidemiology of viral haemorrhagic disease and myxomatosis in a free-living population of wild rabbits. *The Veterinary Record* **150**, 776-782.

Cammisa-Parks, H., Cisar, L. A., Kane, A. & Stollar, V. (1992). The complete nucleotide sequence of cell fusing agent (CFA): homology between the non-structural proteins encoded by CFA and the non-structural proteins encoded by arthropod-borne flaviviruses. *Virology* **189**, 511-524.

Cancellotti, F. M. & Renzi, M. (1991). Epidemiology and current situation of viral haemorrhagic disease of rabbits and the European brown hare syndrome in Italy. *Revue Scientifique Et Technique* **10**, 409-422.

Capucci, L., Scicluna, M. T. & Lavazza, A. (1991). Diagnosis of viral haemorrhagic disease of rabbits and the European brown hare syndrome. *Revue Scientifique Et Technique (International Office of Epizootics)* **10**, 347-370.

Capucci, L., Chasey, D., Lavazza, A. & Westcott, D. (1996a). Preliminary characterization of a non-haemagglutinating strain of Rabbit haemorrhagic disease virus from the United Kingdom. *Journal of Veterinary Medicine B* **43**, 245-250.

Capucci, L., Fusi, P., Lavazza, A., Pacciarini, M. L. & Rossi, C. (1996b). Detection and preliminary characterization of a new rabbit calicivirus related to rabbit hemorrhagic disease virus but nonpathogenic. *Journal of Virology* **70**, 8614-8623.

Capucci, L., Nardin, A. & Lavazza, A. (1997). Seroconversion in an industrial unit of rabbits infected with a non-pathogenic rabbit haemorrhagic disease-like virus. *Veterinary Record* **140**, 647-650.

Capucci, L., Fallacara, F., Grazioli, S., Lavazza, A., Pacciarini, M. L. & Brocchi, E. (1998). A further step in the evolution of rabbit hemorrhagic disease virus: the appearance of the first consistent antigenic variant. *Virus Research* **58**, 115-126.

Carman, J. A., Garner, M. G., Catton, M. G., Thomas, S., Westbury, H. A., Cannon, R. M., Collins, B. J. & Tribe, I. G. (1998). Viral haemorrhagic disease of rabbits and human health. *Epidemiology and Infection* **121**, 409-418.

Castanon, S., Marin, M. S., Martin Alonso, J. M., Boga, J. A., Casais, R., Humara, J. M., Ordas, R. J. & Parra, F. (1999). Immunization with potato plants expressing VP60 protein protects against rabbit hemorrhagic disease virus. *Journal of Virology* **73**, 4452-4455.

Chambers, T. J., Halevy, M., Nestorowicz, A., Rice, C. M. & Lustig, S. (1998). West Nile virus envelope proteins: nucleotide sequence analysis of strains differing in mouse neuroinvasiveness. *Journal of General Virology* **79**, 2375-2380.

Chang, H.-H., Shyu, H.-F., Wang, Y.-M., Sun, D.-S., Shyu, R.-H., Tang, S.-S. & Huang, Y.-S. (2002). Facilitation of cell adhesion by immobilised Dengue viral nonstructural protein 1 (NS1): Argenine-Glycine-Aspartic acid structural mimicry within the Dengue viral NS1 antigen. *Journal of Infectious Diseases* **186**, 743-751.

Chapple, P. J. & Lewis, N. D. (1965). Myxomatosis and the rabbit flea. *Nature* **207**, 388-389.

Chasey, D. (1994). Possible origin of rabbit haemorrhagic disease in the United Kingdom. *Veterinary Record* **135**, 496-499.

Chasey, D. (1997). Rabbit haemorrhagic disease: the new scourge of Oryctolagus cuniculus. *Laboratory Animals* **31**, 33-44.

Chasey, D., Lucas, M., Westcott, D. & Williams, M. (1992). European brown hare syndrome in the U.K.; a calicivirus related to but distinct from that of viral haemorrhagic disease in rabbits. *Archives of Virology* **124**, 363-370.

Chasey, D., Trout, R. C. & Edwards, S. (1997a). Susceptibility of wild rabbits (Oryctolagus cuniculus) in the United Kingdom to rabbit haemorrhagic disease (RHD). *Veterinary Research* **28**, 271-276.

Chasey, D., Trout, R. C., Sharp, G. & Edwards, S. (1997b). Seroepidemiology of RHD in wild rabbits in the UK and susceptibility to infection. In *First international symposium on Caliciviruses,* pp. 156-162. Edited by D. Chasey, R. M. Gaskell & I. N. Clarke. Reading, UK: European Society for Veterinary Virology.

Chen, Z. W., Luckay, A., Sodora, D. L., Telfer, P., Reed, P., Gettie, A., Kanu, J. M., Sadek, R. F., Yee, J., Ho, D. D., Zhang, L. & Marx, P. A. (1997). Human immunodeficiency virus type 2 (HIV-2) seroprevalence and characterization of a distinct HIV-2 genetic subtype from the natural range of simian immunodeficiency virus-infected sooty mangabeys. *Journal of Virology* **71**, 3953-3960.

Cohen, L., Kean, K. M., Girard, M. & van der Werf, S. (1996). Effects of p2 cleavage site mutations on poliovirus polyprotein processing. *Virology* **224**, 34-42.

Collery, P. M., Mooney, J., O'Conner, M. & Nowotny, N. (1995). Rabbit haemorrhagic disease in Ireland. *Veterinary Record* **137**, 547.

Collins, B. J., White, J. R., Lenghaus, C., Boyd, V. & Westbury, H. A. (1995). A competition ELISA for the detection of antibodies to rabbit haemorrhagic disease virus. *Veterinary Microbiology* **43**, 85-96.

Cooke, B. D. (1997). Field epidemiology of rabbit calicivirus in Australia. In *First internation symposium on Caliciviruses*, pp. 151-155. Edited by D. Chasey, R. M. Gaskell & I. N. Clarke. Reading, UK: European society for Veterinary Virology.

Cooke, B. D., Robinson, A. J., Merchant, J. C., Nardin, A. & Capucci, L. (2000). Use of ELISAs in field studies of rabbit haemorrhagic disease (RHD) in Australia. *Epidemiology and Infection* **124**, 563-576.

Cooke, B. D. (2002). Rabbit haemorrhagic disease: field epidemiology and the management of wild rabbit populations. *Revue Scientifique Et Technique (International Office of Epizootics)* **21**, 347-358.

Cooke, B. D. & Fenner, F. (2002). Rabbit haemorrhagic disease and the biological control of wild rabbits, *Oryctolagus cuniculus*, in Australia and New Zealand. *Wildlife Research* **29**, 689-706.

Cooke, B. D., McPhee, S., Robinson, A. J. & Capucci, L. (2002). Rabbit haemorrhagic disease: does a pre-existing RHDV-like virus reduce the effectiveness of RHD as a biological control in Australia? *Wildlife Research* **29**, 673-682.

Cooper, P. D., Steiner-Pryor, S., Scotti, P. D. & Delong, D. (1974). On the nature of poliovirus genetic recombinants. *Journal of General Virology* **23**, 41-49.

Corbet, G. B. (1983). A review of classification of the family Leporidae. *Acta Zoologica Fennica* **174**, 11-15.

Corbet, G. B. (1994). Taxonomy and Origins. In *The European Rabbit: The history and biology of a successful colonizer*, pp. 1-7. Edited by H. V. Thompson & C. M. King. Oxford: Oxford University Press.

Crabtree, M. B., Sang, R. C., Stollar, V., Dunster, L. M. & Miller, B. R. (2003). Genetic and phenotypic characterization of the newly described insect flavivirus, Kamiti River virus. *Archives of Virology* **148**, 1095-1118.

Crawley, M. J. (2005). Statistics: A introduction using R. Chichester: Wiley.

Crochu, S., Cook, S., Attoui, H., Charrel, R. N., De Chesse, R., Belhouchet, M., Lemasson, J.-J., de Micco, P. & de Lamballerie, X. (2004). Sequences of flavivirus-related RNA viruses persist in DNA form integrated in the genome of *Aedes* spp. mosquitoes. *Journal of General Virology* **85**, 1971-1980.

Dahourou, G., Guillot, S., Le Gall, O. & Crainic, R. (2002). Genetic recombination in wild-type poliovirus. *Journal of General Virology* **83**, 3103-3110.

Day, M. F., Fenner, F. & Woodroofe, G. M. (1956). Further studies on the mechanism of mosquito transmission of myxomatosis in the European rabbit. *Journal of Hygiene* **54**, 258-283.

Dhabhar, F. S. (2000). Acute stress enhances whilst chronic stress suppresses skin immunity: The role of stress hormones and leukocyte trafficking. *Annals of the New York Academy of Sciences* **917**, 876-893.

Drynov, I. D., Uryvaev, L. V., Nosikov, V. V. & Zhdanov, V. M. (1981). Integration of the genomes of the tick-borne encephalitis virus and of the cell in chronic infection due to this virus and SV40. *Dokl Akad Nauk SSSR* **258**, 1000-1002.

Edwards, G. P., Dobbie, W. & Berman, D. M. (2002a). Population trends in European rabbits and other wildlife of central Australia in the wake of rabbit haemorrhagic disease. *Wildlife Research* **29**, 557-565.

Edwards, G. P., Dobbie, W. & Berman, D. M. (2002b). Warren ripping: its impacts on European rabbits and other wildlife of central Australia amid the establishment of rabbit haemorrhagic disease. *Wildlife Research* **29**, 567-575.

Egger, D., Pasamontes, L., Bolten, R., Boyko, V. & Bienz, K. (1996). Reversible dissociation of the Poliovirus replication complex: functions and interactions of its components in Viral RNA synthesis. *Journal of Virology* **70**, 8675-8683.

Falconar, A. K. I. (1997). The dengue virus nonstructural-1 protein (NS1) generates antibodies to common epitopes on human blood clotting, integrin/adhesin proteins and binds to human endothelial cells: potential impliations in haemorrhagic fever pathogenesis. *Archives of Virology* **142**, 897-916.

Felsenstein, J. (1985). Confidence limits on phylogenies: An approach using the bootstrap. *Evolution* **39**, 783-791.

Fenner, F., Day, M. F. & Woodroofe, G. M. (1956). Epidemiological consequences of the mechanical transmission of myxomatosis by mosquitoes. *Journal of Hygiene* **54**, 284-303.

Fenner, F. & Fantini, B. (1999). Biological control of vertebrate pests: the history of myxomatosis, an epxeriment in evolution. Wallingford: CABI.

Fenner, F. & Marshall, I. D. (1954). Passive immunity in myxomatosis of the European rabbit (*Oryctolagus cuniculus*): the protection conferred on kittens born by immune does. *Journal of Hygiene, Cambridge* **55**, 149-191.

Fenner, F. & Marshall, I. D. (1955). Occurrence of attenuated strains of Myxoma virus in Europe. *Nature* **176**, 782-783.

Fenner, F. & Ross, J. (1994). Myxomatosis. In *The European Rabbit: The history and biology of a successful colonizer*, pp. 205-239. Edited by H. V. Thompson & C. M. King. Oxford: Oxford University Press.

Fenner, F. & Woodroofe, G. M. (1953). The pathogenesis of infectious myxomatosis: The mechanism of infection and the immunological response int he European rabbit (*Oryctolagus cuniculus*). *British Journal of Experimental Pathology* **34**, 400-411.

Fischer, L., Le Gros, F. X., Mason, P. W. & Paoletti, E. (1997). A recombinant canarypox virus protects rabbits against a lethal rabbit hemorrhagic disease virus (RHDV) challenge. *Vaccine* **15**, 90-96.
Fitter, R. S. R. (1959). The ark in our midst. London: Collins.

Flux, J. E. C. (1994). World Distribution. In *The European Rabbit: The history and biology of a successful colonizer*, pp. 8-21. Edited by H. V. Thompson & C. M. King. Oxford: Oxford University Press.

Forrester, N. L., Boag, B., Moss, S. R., Turner, S. L., Trout, R. C., White, P. J., Hudson, P. J. & Gould, E. A. (2003). Long-term survival of New Zealand rabbit haemorrhagic disease virus RNA in wild rabbits revealed by RT-PCR and phylogenetic analysis. *Journal of General Virology* **84**, 3079-3086.

Frolich, K., Klima, F. & Dedek, J. (1998). Antibodies against rabbit hemorrhagic disease virus in free-ranging red foxes from Germany. *Journal of Wildlife Diseases* **34**, 436-442.

Fuller, H. E., Chasey, D., Lucas, M. H. & Gibbens, J. C. (1993). Rabbit haemorrhagic disease in the United Kingdom. *The Veterinary Record* **133**, 611-613.

Fuller, S. J., Wilson, J. C. & Mather, P. B. (1997). Patterns of differentiation among wild rabbit populations *Oryctolagus cuniculus* L. in arid and semiarid ecosystems of north-eastern Australia. *Molecular Ecology* **6**, 145-153.

Gaidamovich, S. Y., Cherednichenko, Y. N. & Zhdanov, V. M. (1978). On the mechanism of the persistence of Lymphocytic Choriomeningitis virus in the continuous cell line Detroit-6. *Intervirology* **9**, 156-161.

Galvani, A. P. & Slatkin, M. (2003). Evaluating plague and smallpox as historical selective pressures for the *CCR5-Δ32* HIV-resistance allele. *Proceedings of the National Academy of Science USA* **100**, 15276-15279.

Gavier Widen, D. & Morner, T. (1991). Epidemiology and diagnosis of the European brown hare syndrome in Scandinavian countries: a review. *Revue Scientifique Et Technique (International Office of Epizootics)* **10**, 453-458.

Gelfi, J., Chantel, J., Phong, T. T., Py, R. & Boucraut-Baralon, C. (1999). Development of an ELISA for detection of myxoma virus-specific rabbit antibodies: test evaluation for diagnostic applications on vaccinated and wild rabbits sera. *Journal of Veterinary Diagnostic Investigation* **11**, 240-245.

Gibb, J. A. & Williams, J. M. (1994). The rabbit in New Zealand. In *The European Rabbit: The history and biology of a successful colonizer*, pp. 158-204. Edited by H. V. Thompson & C. M. King. Oxford: Oxford University Press.

Goodfellow, I. G., Chaudhry, Y., Richardson, A., Meredith, J. M., Almond, J. W., Barclay, W. S. & Evans, D. J. (2000). Identification of a *cis*-acting replication element (CRE) within the poliovirus encoding region. *Journal of Virology* **74**, 4590-4600.

Gorski, J., Mizak, B. & Chroboci„ska, M. (1994). Control of viral haemorrhagic disease of rabbits in Poland. *Revue Scientifique Et Technique* **13**, 881-891.

Gould, A. R., Kattenbelt, J. A., Lenghaus, C., Morrissy, C., Chamberlain, T., Collins, B. J. & Westbury, H. A. (1997). The complete nucleotide sequence of rabbit haemorrhagic disease virus (Czech strain V351): use of the polymerase chain reaction to detect replication in Australian vertebrates and analysis of viral population sequence variation. *Virus Research* **47**, 7-17.

Gould, E. A. & Buckley, A. (1989). Antibody-dependent enhancement of yellow fever and Japanese encephalitis virus neurovirulence. *Journal of General Virology* **70**, 1605-1608.

Gould, E. A., Chanas, A. C., Buckley, A. & Clegg, C. S. (1983). Monoclonal immunoglobulin M antibody to Japanese encephalitis virus that can react with a nuclear antigen in mammalian cells. *Infection and Immunity* **41**, 774-779.

Graham, D. A., Cassidy, J., Beggs, N., Curran, W. L., McLaren, I. E., Connor, T. J. & Kennedy, S. (1996). Rabbit viral haemorrhagic disease in Northern Ireland. *Veterinary Record* **138**, 47.

Granzow, H., Weiland, F., Strebelow, H. G., Liu, C. M. & Schirrmeier, H. (1996). Rabbit hemorrhagic disease virus (RHDV): ultrastructure and biochemical studies of typical and core-like particles present in liver homogenates. *Virus Research* **41**, 163-172.

Green, R. G., Larson, C. L. & Bell, J. F. (1939). Shock disease as the cause of the periodic decimation of the Snowshoe Hare. *American Journal of Hygiene* **30 section B**, 83-102.

Gregg, D. A., House, C., Meyer, R. & Berninger, M. (1991). Viral haemorrhagic disease of rabbits in Mexico: epidemiology and viral characterization. *Revue Scientifique Et Technique* **10**, 435-451.

Gritsun, T. S., Desai, A. & Gould, E. A. (2001). The degree of attenuation of tick-borne encephalitis virus depends on the cumulative effects of point mutations. *Journal of General Virology* **82**, 1667-1675.

Guittre, C., Ruvoen Clouet, N., Barraud, L., Cherel, Y., Baginski, I., Prave, M., Ganiere, J. P., Trepo, C. & Cova, L. (1996). Early stages of rabbit haemorrhagic disease virus infection monitored by polymerase chain reaction. *Journal of Veterinary Medicine B* **43**, 109-118.

Hahn, C. S., Lustig, S., Strauss, E. G. & Strauss, J. H. (1988). Western equine encephalitis virus is a recombinant virus. *Proceedings of the National Academy of Science USA* **85**, 5997-6001.

Hahn, H. & Palmanberg, A. C. (1996). Mutational analysis of the Encephalomyocarditis virus primary cleavage. *Journal of Virology* **70**, 6870-6875.

Hahn, H. & Palmanberg, A. C. (2001). Deletion mapping of the Encephalomyocarditis virus primary cleavage site. *Journal of Virology* **75**, 7215-7218.

Halasz, R., Weiland, O. & Sallberg, M. (2001). GB virus C/hepatitis G virus. *Scandinavian Journal of Infectious diseases* **33**, 572-580.

Hall, T. A. (1999). BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series* **41**, 95-98.

Halstead, S. B. (1989). Antibody, macrophages, dengue virus infection, shock, and hemorrhage: a pathogenic cascade. *Reviews in Infectious Diseases* **11**, S830-839.

Halstead, S. B. (2003). Neutralization and antibody-dependent enhancement of dengue viruses. *Advances in Virus Research* **60**, 421-467.

Halstead, S. B. & Simasthien, P. (1970). Observations related to the pathogenesis of dengue hemorrhagic fever. II. Antigenic and biologic properties of dengue viruses and their association with disease response in the host. *The Yale Journal of Biology and Medicine* **42**, 276-292.

Hardy, M. E., Kramer, S. F., Treanor, J. J. & Estes, M. K. (1997). Human calicivirus genogroup II capsid sequence diversity revealed by analyses of the prototype Snow Mountain agent. *Archives of Virology* **142**, 1469-1479.

Heath, A. C. G., Heyward, R. P. & Norbury, G. L. (1999). Blowflies (Calliphoridae) as potential vectors of Rabbit haemorrhagic disease virus in New Zealand. In *Rabbit control, (RCD): Dilemmas and Implications. Proceedings of the conference*, pp. 85-88. Wellington, New Zealand: Royal Society of New Zealand.

Heneidi Zeckua, A., Zepeda Sein, C., Mateos Poumian, A. & Velazquez, G. (1997). [Model for evaluating the risk of introducing rabbit viral hemorrhagic disease based on experience in Mexico]. *Revue Scientifique Et Technique* **16**, 91-103.

Henning, J. (2003). Factors influencing the epidemiology of *Rabbit haemorrhagic disease virus* in New Zealand. In *EpiCentre, Institute of Veterinary, Animal and Biological Sciences*, pp. 285. Palmerston North: Massey University.

Henzell, R. P., Cunningham, R. B. & Neave, H. M. (2002). Factors affecting the survival of Australian wild rabbits exposed to rabbit haemorrhagic disease. *Wildlife Research* **29**, 523-542.

Hey, T. D., Richards, O. C. & Ehrenfeld, E. (1986). Synthesis of plus- and minus-strand RNA from Poliovirion RNA template in vitro. *Journal of Virology* **58**, 790-796.

Hudson, J. R. & Mansi, W. (1955). Attenuated strains of myxoma virus in Britain. *Veterinary Record* **67**, 746.

Hudson, J. R., Thompson, H. V. & Mansi, W. (1955). Myxoma virus in Britain. *Nature* **176**, 783.

Irwin, M. (1993). Stress-induced immune suppression: Role of the autonomic nervous system. *Annals of the New York Academy of Sciences* **697**, 203-218.

Johnson, K. M. & Martin, D. H. (1974). Venezuelan equine encephalitis. *Advances in Veterinary Science and Comparative Medicine.* **18**, 79-116.

Joost Haasnoot, P. C., Olsthoorn, R. C. & Bol, J. F. (2002). The Brome mosaic virus subgenomic promoter hairpin is structurally similar to the iron-responsive element and functionally equivalent to the minus-strand core promoter stem-loop C. *RNA* **8**, 110-112.

Joubert, P., Pautigny, C., Madelaine, M. F. & Rasschaert, D. (2000). Identification of a new cleavage site of the 3C-like protease of Rabbit haemorrhagic disease virus. *Journal of General Virology* **81**, 481-488.

Jung, J. Y., Lee, B. J., Tai, J. H., Park, J. H. & Lee, Y. S. (2000). Apoptosis in Rabbit haemorrhagic disease. *Journal of Comparative Pathology* **123**, 135-140.

Katayama, K., Shirato-Horikoshi, H., Kojima, S., Kageyama, T., Oka, T., Hoshino, F. B., Fukushi, S., Shinohara, M., Uchida, K., Suzuki, Y., Gojobori, T., & Takeda, N. (2002). Phylogenetic analysis of the complete genome of 18 Norwalk-like viruses. *Virology* **299**, 225-239.

Kimura, M. (1968). Evolutionary rate at the molecular level. *Nature* **217**, 624-626.

Kimura, T., Mitsui, I., Okada, Y., Furuya, T., Ochiai, K., Umemura, T. & Itakura, C. (2001). Distribution of rabbit haemorrhagic disease virus RNA in experimentally infected rabbits. *Journal of Comparative Pathology* **124**, 134-141.

King, L. & Jukes, T. (1969). Non-darwinian evolution. *Science* **164**, 788-789.

Kinney, R. M., Tsuchiya, K. R., Sneider, J. M. & Trent, D. W. (1992a). Genetic evidence that epizootic Venezuelan equine encephalitis (VEE) viruses may have evolved from enzootic VEE subtype I-D virus. *Virology* **191**, 569-580.

Kinney, R. M., Tsuchiya, K. R., Sneider, J. M. & Trent, D. W. (1992b). Molecular evidence for the origin of the widespread Venezuelan equine encephalitis epizootic of 1969 to 1972. *Journal of General Virology* **73**, 3301-3305.

Klenerman, P., Hengartner, H. & Zinkernagel, R. M. (1997). A non-retroviral RNA virus persists in DNA form. *Nature* **390**, 298-301.

Konig, M., Thiel, H. J. & Meyers, G. (1998). Detection of viral proteins after infection of cultured hepatocytes with rabbit hemorrhagic disease virus. *Journal of Virology* **72**, 4492-4497.

Kovaliski, J. (1998). Monitoring the spread of rabbit hemorrhagic disease virus as a new biological agent for control of wild European rabbits in Australia. *Journal of Wildlife Diseases* **34**, 421-428.

Kpodekon, M. & Alogninouwa, T. (1998). Control of rabbit viral haemorrhagic disease in Benin by vaccination. *Veterinary Record* **143**, 693-694.

Laurent, S., Kut, E., Remy Delaunay, S. & Rasschaert, D. (2002). Folding of the rabbit hemorrhagic disease virus capsid protein and delineation of N-terminal domains dispensable for assembly. *Archives of Virology* **147**, 1559-1571.

Laurent, S., Vautherot, J. F., Le Gall, G., Madelaine, M. F. & Rasschaert, D. (1997). Structural, antigenic and immunogenic relationships between European brown hare syndrome virus and rabbit haemorrhagic disease virus. *Journal of General Virology* **78**, 2803-2811.

Le Gall, G., Arnauld, C., Boilletot, E., Morisse, J. P. & Rasschaert, D. (1998). Molecular epidemiology of rabbit haemorrhagic disease virus outbreaks in France during 1988 to 1995. *Journal of General Virology* **79**, 11-16.

Le Gall, G., Boilletot, E. & Morisse, J. P. (1992). Viral haemorrhagic disease of rabbit: purification and characterization of a strain isolated in France. *Annales de Recherches Veterinaires* **23**, 381-387.

Le Gall, G., Huguet, S., Vende, P., Vautherot, J. F. & Rasschaert, D. (1996). European brown hare syndrome virus: molecular cloning and sequencing of the genome. *Journal of General Virology* **77**, 1693-1697.

Le Gall-Recule, G., Zwingelstein, F., Laurent, S., de Boisseson, C., Portejoie, Y. & Rasschaert, D. (2003). Phylogenetic analysis of rabbit haemorrhagic disease virus in France between 1993 and 2000, and the characterisation of RHDV antigenic varients. *Archives of Virology* **148**, 65-81.

Leighton, A. (1995). Surveillance of wild animal diseases in Europe. *Revue Scientifique Et Technique* **14**, 819-830.

Lenghaus, C., Westbury, H. A., Collins, B. J., Ratnamohan, N. & Morrissy, C. (1994). Overview of the RHD project in Australia. In *Rabbit haemorrhagic disease: Issues in assessment for Biological Control.* Edited by R. K. Munro & R. T. Williams. Canberra: Bureau of Resource Sciences, Australian Government Publishing Service.

Liu, S. J., Xue. H. P., Pu B.Q. & Quian N.H. (1984). A new viral disease in rabbits [in chinese]. *Anim. Husb. Vet. Med.* **16**, 253-255.

Lockley, R. M. (1954). The European rabbit-flea, *Spilopsyllus cuniculi*, as a vector of myxomatosis in Britain. *Veterinary Record* **66**, 434-435.

Lole, K. S., Bollinger, R. C., Paranjape, R. S., Gadkari, D., Kulkarni, S. S., Novak, N. G., Ingersoll, R., Sheppard, H. W. & Ray, S. C. (1999). Full-length human immunodeficiency virus type 1 genomes from subtype C-infected seroconverters in India, with evidence of intersubtype recombination. *Journal of Virology* **73**, 152-160.

Loliger, H. C. & Eskens, U. (1991). Incidence, epizootiology and control of viral haemorrhagic disease of rabbits and the European brown hare syndrome in Germany. *Revue Scientifique Et Technique* **10**, 423-434.

Lopez Vazquez, A. L., Martin Alonso, J. M. & Parra, F. (2001). Characterisation of the RNA-dependent RNA polymerase from Rabbit hemorrhagic disease virus produced in Escherichia coli. *Archives of Virology* **146**, 59-69.

Love, R. A., Maegley, K. A., Yu, X., Ferre, R. A., Lingardo, L. K., Diehl, W. & Fuhrman, S. A. (2004). The crystal structure of the RNA-dependant RNA polymerase from human rhinovirus: A duel function target for common cold antiviral therapy. *Structure* **12**, 1533-1544.

Lubinski, J. M., Ransone, L. J. & Dasgupta, A. (1987). Primer-dependent synthesis of covalently linked dimeric RNA molecules by Poliovirus replicase. *Journal of Virology* **61**, 2997-3003.

Lugton, I. W. (1999). A cross-sectional study of risk factors affecting the outcome of rabbit haemorrhagic disease virus releases in New South Wales. *Australian Veterinary Journal* **77**, 322-328.

Machin, A., Martin Alonso, J. M. & Parra, F. (2001). Identification of the amino acid residue involved in Rabbit haemorrhagic disease virus VPg uridylylation. *Journal of Biological Chemistry* **276**, 27787-27792.

Mackenzie, J. M., Jones, M. K. & Westaway, E. G. (1999). Markers for *trans*-golgi membranes and the intermediate compartment localize to induced membranes with distinct replication functions in flavivirus-infected cells. *Journal of Virology* **73**, 9555-9567.

Magliano, D., Marshall, J. A., Bowden, D. S., Vardaxis, N., Meanger, J. & Lee, J.-Y. (1998). Rubella virus replication complexes are virus-modified lysosomes. *Virology* **240**, 57-63.

Marcato, P. S., Bennazi, C. & Vecchi, G. (1988). Infectious necrotic hepatitis of rabbits. Pathogenesis of a new haemorrhagic disease. *Rivista di Coniglicoltura* **25**, 59-64.

Marcato, P. S., Simon, M. C., Girones, O., Muzquiz, J. L., Garcia, J., Ortega, C. & Alonso, J. L. (1991). Clinical and pathological features of viral haemorrhagic disease of rabbits and the European brown hare syndrome. *Revue Scientifique Et Technique (International Office of Epizootics)* **10**, 372-392.

Marchandeau, S., Bertagnoli, S., Peralta, B., Boucraut-Baralon, C., Letty, J. & Reitz, F. (2004). Possible interaction between myxomatosis and calicivirosis related to rabbit haemorrhagic disease affecting the European rabbit. *Veterinary Record* **155**, 589-592.

Marchandeau, S., Chantal, J., Portejoie, Y., Barraud, S. & Chaval, Y. (1998). Impact of viral haemorrhagic disease on a wild population of European rabbits in France. *Journal of Wildlife Diseases* **34**, 429-435.

Marchandeau, S., Chaval, Y. & Le Goff, E. (2000). Prolonged decline in the abundance of wild European rabbits Oryctolagus cuniculus and high immunity level over three years following the arrival of rabbit haemorrhagic disease. *Wildlife Biology* **6**, 141-147.

Marchandeau, S., Le Gall-Recule, G., Bertagnoli, S., Aubineau, J., Botti, G. & Lavazza, A. (2005). Serological evidence for a non-protective RHDV-like virus. *Veterinary Research* **36**, 53-62.

Marin, M. S., Martin Alonso, J. M., Perez Ordoyo Garcia, L. I., Boga, J. A., Arguello Villares, J. L., Casais, R., Venugopal, K., Jiang, W., Gould, E. A. & Parra, F. (1995). Immunogenic properties of rabbit haemorrhagic disease virus structural protein VP60 expressed by a recombinant baculovirus: an efficient vaccine. *Virus Research* **39**, 119-128.

Marshall, I. D. & Regnery, D. C. (1960). Myxomatosis in a Californian brush rabbit (*Sylvilagus bachmani*). *Nature* **199**, 73-74.

Martin Alonso, J. M., Casais, R., Boga, J. A. & Parra, F. (1996). Processing of rabbit hemorrhagic disease virus polyprotein. *Journal of Virology* **70**, 1261-1265.

Martinez-Torrecuadrada, J. L., Cortes, E., Vela, C., Langeveld, J. P., Meloen, R. H., Dalsgaard, K., Hamilton, W. D. & Casal, J. I. (1998). Antigenic structure of the capsid protein of rabbit haemorrhagic disease virus. *Journal of General Virology* **79**, 1901-1909.

Mason, P. W., Bezborodova, S. V. & Henry, T. M. (2002). Identification and characterization of a *cis*-acting element (CRE) adjacent to the internal ribosome entry site of foot-and-mouth disease virus. *Journal of Virology* **76**, 9686-9694.

McColl, K. A., Merchant, J. C., Hardy, J. R., Cooke, B. & Robinson, A. J. (2002a). Evidence for insect transmission of rabbit haemorrhagic disease virus. *Epidemiology and Infection* **129**, 655-663.

McColl, K. A., Morrissy, C. J., Collins, B. J. & Westbury, H. A. (2002b). Persistence of rabbit haemorrhagic disease virus in decomposing rabbit carcases. *Australian Veterinary Journal* **80**, 298-299.

McCulloch, P. & Nelder, J. A. (1989). Generalized Linear Models, 2nd Edn. New York: Chapman & Hall.

Meyers, G. (2003). Translation of the minor capsid protein of a calicivirus is initiated by a novel termination-dependent reinitiation mechanism. *Journal of Biological Chemistry* **278**, 34051-34060.

Meyers, G., Wirblich, C. & Thiel, H. J. (1991a). Genomic and subgenomic RNAs of rabbit hemorrhagic disease virus are both protein-linked and packaged into particles. *Virology* **184**, 677-686.

Meyers, G., Wirblich, C. & Thiel, H. J. (1991b). Rabbit hemorrhagic disease virus - molecular cloning and nucleotide sequencing of a calicivirus genome. *Virology* **184**, 664-676.

Meyers, G., Wirblich, C., Thiel, H. J. & Thumfart, J. O. (2000). Rabbit hemorrhagic disease virus: genome organization and polyprotein processing of a calicivirus studied after transient expression of cDNA constructs. *Virology* **276**, 349-363.

Middleton, A. D. (1934). Periodic fluctuations in British game populations. *Journal of Animal Ecology* **3-4**, 231-245.

Mikami, O., Park, J. H., Kimura, T., Ochiai, K. & Itakura, C. (1999). Hepatic lesions in young rabbits experimentally infected with rabbit haemorrhagic disease virus. *Research in Veterinary Science* **66**, 237-242.

Mizoguchi, T., Itou, K. & Sakurai, M. (2003). Hemagglutination and Antigenic Comparison of Rabbit Haemorrhagic Disease Virus. *Journal of Veterinary Medical Science.* **65**, 95-97.

Monci, F., Sanchez-Campos, S., Navas-Castillo, J. & Moriones, E. (2002). A natural recombinant between the geminiviruses Tomato yellow leaf curl Sardinia virus and Tomato yellow leaf curl virus exhibits a novel pathogenic phenotype and is becoming prevalent in Spanish populations. *Virology* **303**, 317-326.

Morales, M., Barcena, J., Ramirez, M. A., Boga, J. A., Parra, F. & Torres, J. M. (2004). Synthesis *in Vitro* of Rabbit haemorrhagic disease virus subgenomic RNA by internal initiation on (-) sense genomic RNA. *The Journal of Biological Chemistry* **279**, 17013-17018.

Moses, A. (1911). O Virus do mixoma does coelhos. *Memorias do Instituto Oswaldo Crus (Rio de Janeiro)* **3**, 46-53.

Moss, S. R., Turner, S. L., Trout, R. C., White, P. J., Hudson, P. J., Desai, A., Armesto, M., Forrester, N. L. & Gould, E. A. (2002). Molecular epidemiology of Rabbit haemorrhagic disease virus. *Journal of General Virology* **83**, 2461-2467.

Motha, M. X. J. & R.G.Clark (1998). Confirmation of Rabbit Haemorrhagic Disease in wild New Zealand Rabbits using the ELISA. *New Zealand Veterinary Journal* **46**, 83-84.

Muirhead-Thompson, R. C. (1956). The part played by woodland mosquitoes of the genus *Aedes* in the transmission of myxomatosis in England. *Journal of Hygiene* **54**, 461-471.

Mutze, G., Bird, P., Kovaliski, J., Peacock, D., Jennings, S. & Cooke, B. (2002). Emerging epidemiological patterns in rabbit haemorrhagic disease, its interaction with myxomatosis and their effects on rabbit populations in South Australia. *Wildlife Research* **20**, 577-590.

Mutze, G., Cooke, B. & Alexander, P. (1998). The initial impact of Rabbit haemorrhagic disease on European rabbit populations in South Australia. *Journal of Wildlife Diseases* **34**, 221-227.

Myers, K. (1954). Studies in the epidemiology of infectious myxomatosis of rabbits. II. Field experiments, August-November 1950, and the first epizootic of myxomatosis in the riverine plain of south-eastern Australia. *Journal of Hygiene* **52**, 47-59.

Myers, K., Parer, I., Wood, D. & Cooke, B. D. (1994). The rabbit in Australia. In *The European Rabbit: The history and biology of a successful colonizer*, pp. 108-157. Edited by H. V. Thompson & C. M. King. Oxford: Oxford University Press.

Nagesha, H. S., McColl, K. A., Collins, B. J., Morrissy, C. J., Wang, L. F. & Westbury, H. A. (2000). The presence of cross-reactive antibodies to rabbit haemorrhagic disease virus in Australian wild rabbits prior to the escape of virus from quarantine. *Archives of Virology* **145**, 749-757.

Nagy, P. D. and Simon, A. E. (1997). New insights into the mechanisms of RNA recombination. *Virology* **235**,1-9.

Neufeld, K. L., Richards, O. C. & Ehrenfeld, E. (1991). Purification, characterization, and comparison of Poliovirus RNA Polymerase from native and recombinant sources. *Journal of Biological Chemistry* **266**, 24212-24219.

Nichol, S. T., Rowe, J. E. & Fitch, W. M. (1993). Punctuated equilibrium and positive Darwinian evolution in vesicular stomatitis virus. *Proceedings of the National Academy of Science USA* **90**, 10424-10428.

Nowotny, N., Bascunana, C. R., Ballagi Pordany, A., Gavier Widen, D., Uhlen, M. & Belak, S. (1997). Phylogenetic analysis of rabbit haemorrhagic disease and European brown hare syndrome viruses by comparison of sequences from the capsid protein gene. *Archives of Virology* **142**, 657-673.

Nowotny, N., Schilcher, F., Fuchs, A. & Loupal, G. (1992). Zum Aufhreten der Rabbit Haemorrhagic Disease (RHD) in Österreich:II Epizootiology Unter Suchungen. *Wien Tierärztl Mschr* **79**, 134-140.

Ohlinger, V. F., Haas, B., Meyers, G., Weiland, F. & Thiel, H. J. (1990). Identification and characterization of the virus causing rabbit hemorrhagic disease. *Journal of Virology* **64**, 3331-3336.

Ohlinger, V. F. & Thiel, H. J. (1991). Identification of the viral haemorrhagic disease virus of rabbits as a calicivirus. *Revue Scientifique Et Technique* **10**, 311-323.

O'Keefe, J. S., Temparo, J. E., Atkinson, P. H., Pacciarini, M. L., Fallacara, F., Horner, G. & Motha, M. X. J. (1998). Typing of Rabbit Haemorrhagic Disease Virus from New Zealand Wild Rabbits. *New Zealand Veterinary Journal* **46**, 41-42.

O'Keefe, J. S., Tempero, J. E., Motha, M. X., Hansen, M. F. & Atkinsona, P. H. (1999). Serology of rabbit haemorrhagic disease virus in wild rabbits before and after release of the virus in New Zealand. *Veterinary Microbiology* **66**, 29-40.

Oldstone, M. B. A. (1987). Molecular mimicry and autoimmune disease. *Cell* **50**, 819-820.

Oliver, S. L., Brown, D. W. G., Green, J. & Bridger, J. C. (2004). A chimeric bovine enteric calicivirus: evidence for genomic recombination in genogroup III of the *Norovirus* genus of the *Caliciviridae*. *Virology* **326**, 231-239.

Park, J. H., Lee, Y. S. & Itakura, C. (1995). Fibrin(ogen)-related antigens in rabbits experimentally infected with rabbit haemorrhagic disease virus. *Research in Veterinary Science* **63**, 123-127.

Park, N. Y., Chong, C. Y., Kim, J. H., Cho, S. M., Cha, Y. H., Jung, B. T., Kim, D. S. & Yoon, J. B. (1987). An outbreak of haemorrhagic pneumonia of rabbits in Korea. *Journal of the Korean Medical Association* **23**, 603-710.

Parkes, J. P., Norbury, G. L., Heyward, R. P. & Sullivan, G. (2002). Epidemiology of rabbit haemorrhagic disease (RHD) in the South Island, New Zealand, 1997-2001. *Wildlife Research* **29**, 543-555.

Parra, F., Boga, J. A., Marin, M. S. & Casais, R. (1993). The amino acid terminal sequence of VP60 from rabbit haemorrhagic disease virus supports its putative subgenomic origin. *Virus Research* **27**, 219-228.

Parra, F. & Prieto, J. M. (1990). Purification and characterization of a calicivirus as the causative agent of a lethal hemorrhagic disease in rabbits. *Journal of Virology* **64**, 4013-4015.

Patterson, I. A. & Howie, F. E. (1995). Rabbit haemorrhagic disease in Scotland. *Veterinary Record* **137**, 523.

Pelletier, J. & Sonenburg, N. (1988). Internal initiation of translation of eukaryotic mRNA directed by a sequence derived from poliovirus RNA. *Nature* **334**, 320-325.

Plana Duran, J., Bastons, M., Rodriguez, M. J., Climent, I., Cortes, E., Vela, C. & Casal, I. (1996). Oral immunization of rabbits with VP60 particles confers protection against rabbit hemorrhagic disease. *Archives of Virology* **141**, 1423-1436.

Plotch, S. J., Palant, O. & Gluzman, Y. (1989). Purification and properties of Poliovirus RNA polymerase expressed in *Escherichia coli*. *Journal of Virology* **63**, 216-225.

Posada, D. C. & Crandall, K. A. (1998). MODELTEST: testing the model of DNA substitution. *Bioinformatics* **14**, 817-818.

Prieto, J. M., Fernandez, F., Alvarez, V., Espi, A., Garcia Marin, J. F., Alvarez, M., Martin, J. M. & Parra, F. (2000). Immunohistochemical localisation of rabbit haemorrhagic disease virus VP-60 antigen in early infection of young and adult rabbits. *Research in Veterinary Science* **68**, 181-187.

Ramiro Ibanez, F., Martin Alonso, J. M., Garcia Palencia, P., Parra, F. & Alonso, C. (1999). Macrophage tropism of rabbit hemorrhagic disease virus is associated with vascular pathology. *Virus Research* **60**, 21-28.

Rasschaert, D., Huguet, S., Madelaine, M. F. & Vautherot, J. F. (1995). Sequence and genomic organization of a rabbit hemorrhagic disease virus isolated from a wild rabbit. *Virus Genes* **9**, 121-132.

Ratcliffe, F. N., Myers, K., Fennessy, B. V. & Calaby, J. H. (1952). Myxomatosis in Australia. A step towards the biological control of the rabbit. *Nature* **170**, 7-11.

Ray, S. C. (1997). SimPlot for Windows, version 3.5.1. [Online]. Baltimore, Md.: (Distributed by Author). http://sray.med.som.jhmi.edu/SCRoftware/

Regnery, D. C. & Miller, J. H. (1972). A myxoma virus epizootic in a brush rabbit population. *Journal of Wildlife Research* **8**, 327-331.

Restrepo-Hartwig, M. A. & Ahlquist, P. (1996). Brome Mosaic Virus helicase - and polymerase-like proteins colocalize on the endoplasmic reticulum at sites of viral RNA synthesis. *Journal of Virology* **70**, 8908-8916.

Rico-Hesse, R., Roehrig, J. T., Trent, D. W. & Dickerman, R. W. (1988). Genetic variation of Venezuelan equine encephalitis virus strains of the ID variety in Colombia. *American Journal of Tropical Medicine and Hygiene* **38**, 195-204.

Robinson, A. J., Kirkland, P. D., Forrester, R. I., Capucci, L., Cooke, B. D. & Philbey, A. W. (2002a). Serological evidence for the presence of a calicivirus in Australian wild rabbits, *Oryctolagus cuniculus*, before the introduction of rabbit haemorrhagic disease virus (RHDV): its potential influence on the specificity of a competitive ELISA for RHDV. *Wildlife Research* **29**, 655-662.

Robinson, A. J., So, P. T. M., Müller, W. J., Cooke, B. D. & Capucci, L. (2002b). Statistical models for the effect of age and maternal antibodies on the development of rabbit haemorrhagic disease in Australian wild rabbits. *Wildlife Research* **29**, 663-671.

Rodak, L., Smid, B. & Valicek, L. (1991). Application of control measures against viral haemorrhagic disease of rabbits in the Czech and Slovak Federal Republic. *Revue Scientifique Et Technique* **10**, 513-524.

Rodak, L., Smid, B., Valicek, L., Vesely, T., St•panek, J., Hampl, J. & Jurak, E. (1990). Enzyme-linked immunosorbent assay of antibodies to rabbit haemorrhagic disease virus and determination of its major structural proteins. *Journal of General Virology* **71**, 1075-1080.

Rogers, P. M., Arthur, C. P. & Soriguer, R. C. (1994a). The rabbit in continental Europe. In *The European Rabbit: The history and biology of a successful colonizer*, pp. 22-63. Edited by H. V. Thompson & C. M. King. Oxford: Oxford University Press.

Ross, J. (1982). Myxomatosis: the natural evolution of the disease. *Symposium of the Zoological Society of London* **50**, 77-95.

Ross, J., Tittensor, A. M., A.P., F. & Sanders, M. F. (1989). Myxomatosis in farmland rabbit populations in England and Wales. *Epidemiology and Infection* **103**, 333-357.

Ross, J. R. & Sanders, M. F. (1977). Innate resistance of myxomatosis in wild rabbits in England. *Journal of Hygiene* **79**, 411-415.

Ross, J. R. & Sanders, M. F. (1984). The development of genetic resistance to myxomatosis in wild rabbits in Britain. *Journal of Hygiene* **92**, 255-261.

Ross, J. R. & Sanders, M. F. (1987). Changes in the virulence of myxoma strains in Britain. *Epidemiology and Infection* **98**, 113-117.

Ryabchikova, E. I. & Price, B. B. S. (2004). Ebola and Marburg viruses: A view of infection using electron microscopy. Columbus: Battelle Press

Ryan, M. D. & Drew, J. (1994). Foot-and-mouth disease virus 2A oligopeptide mediated cleavage of an artificial polyprotein. *EMBO Journal* **13**, 928-933.

Ryan, M. D., King, A. M. Q. & Thomas, P. (1991). Cleavage of foot-and-mouth disease virus polyprotein is mediated by residues located within a 19 amino acid sequence. *Journal of General Virology* **72**, 2727-2732.

Saint, K. M., French, N. & Kerr, P. J. (2001). Genetic variation in Australian isolates of myxoma virus: an evolutionary and epidemiological study. *Archives of Virology* **146**, 1105-1123.

Sallie, R. (2005). Replicative Homeostasis: A fundamental mechanism mediating selective viral replication and escape mutation. *Virology Journal* **2**, 10.

Salminen, M. O., Carr, J. K., Burke, D. S. & McCutchan, F. E. (1995). Identification of breakpoints in intergenotypic recombinants of HIV type 1 by bootscanning. *AIDS Research in Human Retroviruses* **11**, 1423-1425.

Sammin, D. J., Markey, B. K. & Bassett, H. F. (1995). Rabbit haemorrhagic disease in Ireland. *Veterinary Record* **137**, 599-600.

Sanarelli, G. (1898). Das myxomatogene Virus. Beitrag zum Stadium der Krankheitserreger asserhalb des Sichtbaren. *Zentralblatt für Bakteriologie, Parasitenkunde, Infektionskrankheiten und Hygiene (Abt.1)* **23**, 865-873.

Sandell, P. R. (2002a). Implications of rabbit haemorrhagic disease for the short-term recovery of semi-arid woodland communities in north-west Victoria. *Wildlife Research* **29**, 591-598.

Sang, R. C., Gichogo, A., Gachoya, J., Dunster, M. D., Ofula, V., Hunt, A. R., Crabtree, M. B., Miller, B. R. & Dunster, L. M. (2003). Isolation of a new flavivirus related to cell fusing agent virus (CFAV) from field collected flood-water *Aedes* mosquitoes sampled from a dambo in central Kenya. *Archives of Virology* **148**, 1085-1093.

Santti, J., Hyypia, T., Kinnunen, L. & Salminen, M. (1999). Evidence of recombination among Enteroviruses. *Journal of Virology* **73**, 8741-8749.

Schaad, M. C., Jensen, P. E. & Carrington, J. C. (1997). Formation of plant RNA virus replication complexes on membranes: role of an endoplasmic reticulum-targeted viral protein. *The EMBO Journal* **16**, 4049-4059.

Schirrmeier, H., Granzow, H. & Kollner, B. (1997). The variability of rabbit haemorrhagic disease virus (RHDV) and its relation to other caliciviruses. In *Proceedings of the First International Symposium of Caliciviruses ESVV*, pp. 194-202. Edited by D. Chasey, R. M. Gaskell & I. N. Clarke.

Schirrmeier, H., Reimann, I., Kollner, B. & Granzow, H. (1999). Pathogenic, antigenic and molecular properties of rabbit haemorrhagic disease virus (RHDV) isolated from vaccinated rabbits: detection and characterization of antigenic variants. *Archives of Virology* **144**, 719-735.

Seal, B. S., Neill, J. D. & Ridpath, J. F. (1994). Predicted stem-loop structures and variation in nucleotide sequence of 3' noncoding regions among animal calicivirus genomes. *Virus Genes* **8**, 243-247.

Sharp, P. M., Bailes, E., Robertson, D. L., Gao, F. & Hahn, B. H. (1999). Origin and evolution of AIDS viruses. *The Biological Bulletin* **196**, 338-342.

Sharp, P. M., Robertson, D. L. & Hahn, B. H. (1995). Cross-species transmission and recombination of "AIDS" viruses. *Philosophical Transactions of the Royal Society of London. Series B: Biological Sciences* **349**, 41-47.

Sheail, J. (1971). Rabbits and their history. Newton Abbot: David and Charles.

Sheail, J. (1978). Rabbits and agriculture in post-medieval England. *Journal of Historical Geography* **4**, 343-355.

Sheail, J. (1991). The management of an animal population: changing attitudes towards the wild rabbit in Britain. *Journal of Environmental Management* **33**, 189-203.

Shien, J. H., Shieh, H. K. & Lee, L. H. (2000). Experimental infections of rabbits with Rabbit haemorrhagic disease virus monitored by polymerase chain reaction. *Research in Veterinary Science* **68**, 255-259.

Shirato, K., Miyoshi, H., Goto, A., Ako, Y., Ueko, T., Kariwa, H. & Takashima, I. (2004). Viral envelope protein glycosylation is a molecular determinant of the neuroinvasiveness of the New York strain of West Nile virus. *Journal of General Virology* **85**, 3637-3645.

Shlegel, A., Giddings, T. H., Ladinsky, M. S. & Kirkegaard, K. (1996). Cellular origin and ultrastructure of membranes induced during Poliovirus infection. *Journal of Virology* **70**, 6576-6588.

Simmonds, P. & Smith, D. B. (1999). Structural constraints on RNA virus evolution. *Journal of Virology* **73**, 5787-5794.

Simmonds, P., Tuplin, A. & Evans, D. J. (2004). Detection of genome-scale ordered RNA structure (GORS) in genomes of postive-stranded RNA viruses: Implications for virus evolution and host persistence. *RNA* **10**, 1337-1351.

Simon, M. C., Girones, O., Muguruza, R., Alonso, J. L., Muzquiz, J. L. & Ortega, C. (1995). Diagnostic survey of viral haemorrhagic disease in wild rabbits (*Oryctolagus cuniculus*) in four regions of Spain. *Revue Scientifique Et Technique* **14**, 789-799, 801-810.

Simon, M. C., Ortega, C., Maynar, P., Muzquiz, J. L., De Blas, I., Girones, O., Alonso, J. L. & Sanchez, J. (1998). Studies in wild rabbit (*Oryctolagus cuniculus*) populations in Navarra (Spain) I. Epidemiology of rabbit viral haemorrhagic disease. *Gibier Faune Sauvage, Game Wildlife* **15**, 47-64.

Simpson, J. (1893). The wild rabbit in a new aspect; or, Rabbit-warrens that pay. Edinburgh.

Smid, B., Valicek, L., Stepanek, J., Jurak, E. & Rodak, L. (1989). Experimental transmission and electron microscopic demonstration of the virus of haemorrhagic disease of rabbits in Czechoslovakia. **36**, 237-240.

Smith, D. B. & Simmonds, P. (1997). Characteristics of nucleotide substitution in the Hepatitis C virus genome: constraints on sequence change in coding regions at both ends of the genome. *Journal of Molecular Evolution* **45**, 238-246.

Soriguer, R. C. & Myers, K. (1986). Morphological, physiological and reproductive features of a wild rabbit population in Mediterranean Spain under different habitat management. *Mammal Review* **16**, 197.

Steinhauer, D. A., Domingo, E. & Holland, J. J. (1992). Lack of evidence for proofreading mechanisms associated with an RNA virus polymerase. *Gene* **122**, 281-288.

Stephens, M. N. (1952). Seasonal observations on the wild rabbit (*Oryctolagus cuniculus cuniculus L.*) in West Wales. *Proceedings of the Zoological Society, London* **122**, 417-434.

Strauss, J. H. & Strauss, E. G. (1994). The alphaviruses: gene expression, replication, and evolution. *Microbiology Reviews* **58**, 491-562.

Sullivan, J., Abdo, Z., Joyce, P. & Swofford, D. (2005). Evaluating the performance of a successive -approximations approach to parameter optimization in Maximum-Likelihood phylogeny estimation. *Molecular Biology and Evolution* **22**, 1386-1392.

Sumption, K. J. & Flowerdew, J. R. (1985). The ecological effects of the decline in rabbits due to Myxomatosis. *Mammal Review* **15**, 151-186.

Surridge, A. K., Bell, D. J., Ibrahim, K. M. & Hewitt, G. M. (1999a). Population structure and genetic variation of European wild rabbits (*Oryctolagus cuniculus*) in East Anglia. *Heredity* **82**, 479-487.

Surridge, A. K., Ibrahim, K. M., Bell, D. J., Webb, N. J., Rico, C. & Hewitt, G. M. (1999b). Fine-scale genetic structuring in a natural population of European wild rabbits. *Molecular Ecology* **8**, 299-307.

Sutcliffe, A. J. (1964). The mammalian fauna. In *The Swanscombe skull. A survey of research on a Pleistocene site*, pp. 85-112. Edited by C. D. Ovey.

Swofford, D. (2000). PAUP*. Phylogenetic Analysis Using Parsimony (* and other methods). Version 4. Sunderland, Mass.: Sinauer Associates.

Takamatsu, H., Mellor, P. S., Mertens, P. P. C., Kirkham, P. A., Burroughs, J. N. & Parkhouse, R. M. E. (2003). A possible overwintering mechanism for Bluetongue virus in the absence of the insect vector. *Journal of General Virology* **84**, 227-235.

Tapper, S. C. (1992). Game heritage: an ecological review from shooting and gamekeeping records. Fordingbridge: Game Conservancy.

Teifke, J. P., Reimann, I. & Schirrmeier, H. (2002). Subacute liver necrosis after experimental infection with Rabbit haemorrhagic disease virus (RHDV). *Journal of Comparative Pathology* **126**, 231-234.

Thiel, H. J. & Konig, M. (1999). Caliciviruses: an overview. *Veterinary Microbiology* **69**, 55-62.

Thompson, H. V. (1994). The rabbit in Britain. In *The European Rabbit: The history and biology of a successful colonizer*, pp. 64-107. Edited by H. V. Thompson & C. M. King. Oxford: Oxford University Press.

Thompson, H. V. & Worden, A. N. (1956). The rabbit. London: Collins New Naturalist.

Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F. & Higgins, D. G. (1997). The CLUSTAL X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research* **15**, 4876-4882.

Thouvenin, E., Laurent, S., Madelaine, M. F., Rasschaert, D., Vautherot, J. F. & Hewat, E. A. (1997). Bivalent binding of a neutralising antibody to a calicivirus involves the torsional flexibility of the antibody hinge. *Journal of Molecular Biology* **270**, 238-246.

Thumfart, J. O. & Meyers, G. (2002). Rabbit haemorrhagic disease virus: Identification of a cleavage site in the viral polyprotein that is not processed by the known calicivirus protease. *Virology* **304**, 352-363.

Torres, J. M., Sanchez, C., Ramirez, M. A., Morales, M., Barcena, J., Ferrer, J., Espuna, E., Pages Mante, A. & Sanchez Vizcaino, J. M. (2001). First field trial of a transmissible recombinant vaccine against myxomatosis and rabbit hemorrhagic disease. *Vaccine* **19**, 4536-4543.

Trout, R. C. (2003). Rabbits in the farmland ecosystems. In *Conservation and Conflict: Mammals and Farming in BritainProceedings of a joint conference of the Mammal Society and Linnean Society*, pp. 198-210. Edited by F. Tattersall & W. Manley. Otley: Westbury Publishing.

Trout, R. C., Chasey, D., Holloway, S. & Bullock, I. (1997a). The national and local rate of spread of rabbit haemorrhagic disease virus in Britain. In *First International Symposium on Caliciviruses*, pp. 163-171. Edited by D. Chasey, R. M. Gaskell & I. N. Clarke. Reading, UK: European Society for Veterinary Virology.

Trout, R. C., Chasey, D. & Sharp, G. (1997b). Seroepidemiology of rabbit haemorrhagic disease (RHD) in wild rabbits (*Oryctolagus cuniculus*) in the United Kingdom. *Journal of Zoology London* **243**, 846-853.

Trout, R. C., Ross, J., Tittensor, A. M. & Fox, A. P. (1992). The effect on a British wild rabbit population (*Orcyctolagus cuniculus*) of manipulating myxomatosis. *Journal of Applied Ecology* **29**, 679-686.

Trout, R. C., Tapper, S. C. & Harradine, J. (1986). Recent trends in the rabbit population in Britain. *Mammal Review* **16**, 117-123.

Tsukiyama Kohara, K., Iizuka, N., Kohara, M. & Nomoto, A. (1992). Internal ribosome entry site within hepatitis C virus RNA. *Journal of Virology* **66**, 1476-1483.

Tunon, M. J., Sanchez-Campos, S., Garcia-Ferreras, J., Alvarez, M., Jorquera, F. & Gonzalez-Gallego, J. (2003). Rabbit hemorrhagic viral disease: Characterization of a new animal model of fulminant liver failure. *Journal of Laboratory and Clinical Medicine* **141**, 272-278.

Twiddy, S. S. & Holmes, E. C. (2003). The extent of homologous recombination in members of the genus *Flavivirus*. *Journal of General Virology* **84**, 429-440.

Uchil, P. D. & Satchidanandam, V. (2003a). Architecture of the flaviviral replication complex. *The Journal of Biological Chemistry* **278**, 24388-24398.

Uchil, P. D. & Satchidanandam, V. (2003b). Characterization of RNA synthesis, replication mechanism, and in vitro RNA-dependent RNA polymerase activity of Japanese encephalitis virus. *Virology* **307**, 358-371.

Ueda, K., Park, J. H., Ochiai, K. & Itakura, C. (1992). Disseminated intravascular coagulation (DIC) in rabbit haemorrhagic disease. *Japanese Journal of Veterinary Research* **40**, 133-141.

Uzcategui, N. Y., Camacho, D., Comach, G., Cuello de Uzacategui, R., Holmes, E. C. & Gould, E. A. (2001). Molecular epidemiology of dengue type 2 virus in Venezuela: evidence for *in situ* virus evolution and recombination. *Journal of General Virology* **82**, 2945-2953.

van der Meer, Y., van Tol, H., Krijnse Locker, J. & Snijder, E. J. (1998). ORF1a-encoded replicase subunits are involved in the membrane association of Arterivirus replication complex. *Journal of Virology* **72**, 6689-6698.

Vandamme, A.-M. (2003). Basic concepts of Molecular Evolution. In *The Phylogenetic Handbook: a practical approach to DNA and Protein Phylogeny*, pp. 1-23. Edited by M. Salemi & A.-M. Vandamme. Cambridge: Cambridge University Press.

Vaughan, H. E. N. & Vaughan, J. A. (1968). Some aspects of the epizootiology of myxomatosis. *Symposium of the Zoological Society of London* **24**, 289-309.

Veale, E. M. (1957). The rabbit in England. *Agricultural History Review* **5**, 85-90.

Viaplana, E., Plana, J. & Villaverde, A. (1997). Antigenicity of VP60 structural protein of rabbit haemorrhagic disease virus. *Archives of Virology* **142**, 1843-1848.

Villafuerte, R., Calvete, C., Gortazar, C. & Moreno, S. (1994). First epizootic of rabbit haemorrhagic disease in free living populations of *Oryctolagus cuniculus* at Donana national park, Spain. *Journal of Wildlife Diseases* **30**, 176-179.

Walton, T. E. & Grayson, M. A. (1988). Venezuelan equine encephalomyelitis. In *The Arboviruses: Epidemiology and Ecology*, pp. 203-231. Edited by T. P. Monath. Boca Raton: CRC Press.

Weaver, S. C., Kang, W., Shirako, Y., Rumenapf, T., Strauss, E. G. & Strauss, J. H. (1997). Recombinational history and molecular evolution of western encephalomyelitis complex alphaviruses. *Journal of Virology* **71**, 613-623.

Westaway, E. G. (1987). Flavivirus replication strategy. *Advances in Virus Research* **33**, 45-90.

Westaway, E. G., Khromykh, A. A. & Mackenzie, J. M. (1999). Nascent Flavivirus RNA colocalized *in situ* with double-stranded RNA in stable replication complexes. *Virology* **258**, 108-117.

Westaway, E. G., Mackenzie, J. M., Kenney, M. T., Jones, M. K. & Khromykh, A. A. (1997). Ultrastructure of Kunjin virus-infected cells: Co-localization of NS1 and NS3 with double-stranded RNA and NS2B with NS3, in virus-induced membrane structures. *Journal of Virology* **71**, 6650-6661.

White, P. J., Norman, R. A., Trout, R. C., Gould, E. A. & Hudson, P. J. (2001). The emergence of rabbit haemorrhagic disease virus: will a non-pathogenic strain protect the UK? *Philosophical Transactions of the Royal Society of London. Series B: Biological Sciences* **356**, 1087-1095.

White, P. J., Trout, R. C., Moss, S. R., Desai, A., Armesto, M., Forrester, N. L., Gould, E. A. & Hudson, P. J. (2004). Epidemiology of rabbit haemorrhagic disease virus in the United Kingdom: evidence for seasonal transmission by both virulent and avirulent modes of infection. *Epidemiology and Infection* **132**, 555-567.

Wimmer, E., Hellen, C. U. & Cao, X. (1993). Genetics of Poliovirus. *Annual Review of Genetics* **27**, 353-436.

Wirblich, C., Meyers, G., Ohlinger, V. F., Capucci, L., Eskens, U., Haas, B. & Thiel, H. J. (1994). European brown hare syndrome virus: relationship to rabbit hemorrhagic disease virus and other caliciviruses. *Journal of Virology* **68**, 5164-5173.

Wirblich, C., Thiel, H. J. & Meyers, G. (1996). Genetic map of the calicivirus rabbit hemorrhagic disease virus as deduced from in vitro translation studies. *Journal of Virology* **70**, 7974-7983.

Wood, D. H. (1980). The demography of a rabbit population in an arid region of New South Wales, Australia. *Journal of Animal Ecology* **49**, 55-79.

Worobey, M. (2000). Extensive homologous recombination among widely divergent TT viruses. *Journal of Virology* **74**, 7666-7670.

Worobey, M. & Holmes, E. C. (1999). Evolutionary aspects of recombination in RNA viruses. *Journal of General Virology* **80**, 2535-2543.

Xiang, W., Paul, A. V. & Wimmer, E. (1997). RNA signals in Entero- and Rhinovirus genome replication. *Seminars in Virology* **8**, 256-273.

Xu, W. Y. (1991). Viral haemorrhagic disease of rabbits in the People's Republic of China: epidemiology and virus characterisation. *Revue Scientifique Et Technique* **10**, 393-408.

Yin, Y. P., Gregory, V., Bennett, M. & Hay, A. (2004). Recent changes among human influenza viruses. *Virus Research* **103**, 47-52.

Zeuner, F. E. (1963). A history of domesticated animals. London: Hutchinson.

Zeuner, F. E. & Sutcliffe, A. (1964). The excavation of Gorham's cave, Gibraltar, 1951-54:Appendix I. Preliminary report on the Mammalia of Gorham's cave, Gibraltar. *The Bulletin of the Institute of Archaeology* **4**, 213-216.

Zhdanov, V. M. (1975). Integration of viral genomes. *Nature* **256**, 471-473.

Zhdanov, V. M. & Azadova, N. B. (1976). Integration and transfection of an arbovirus by mammalian cells. *Mol Biol (Mosk)* **10**, 1296-1302.

Zhdanov, V. M. & Pafanovich, M. I. (1974). Integration of Measles virus nucleic acid into the cell genome. *Arch Gesamte Virusforsch* **45**, 225-234.

Zheng, T., Napier, A. M., Parkes, J. P., O'Keefe, J. S. & Atkinson, P. H. (2002). Detection of RNA of rabbit haemorrhagic disease virus from New Zealand wild rabbits. *Wildlife Research* **29**, 683-688.