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Rapid Communication

Benign circulation of rabbit haemorrhagic disease virus on Lambay Island, Eire☆

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Abstract

With the exception of virus strains Ashington and RCV, other recognised strains of *Rabbit haemorrhagic disease virus* (RHDV) share relatively close genetic homology. Using serology and phylogenetic analysis, we have identified a third disparate virus lineage in healthy rabbits on Lambay Island off the east coast of Eire, where disease due to RHDV has never been observed. ELISA tests revealed high titre RHDV-specific antibodies in 81% of the sera from 11 healthy rabbits captured on this island, indicating that the virus is actively circulating amongst these rabbits. Nevertheless, infectious virus has not been isolated from rabbits living on this island. The detection of antibodies and the disparate Lambay virus lineage in an apparently healthy and isolated wild rabbit population provides the most convincing evidence yet that at least some strains of RHDV can circulate harmlessly for long periods of time in wild rabbits possibly by producing persistent or latent infections.

Keyword: Rabbit haemorrhagic disease virus

Introduction

The emergence of *Rabbit haemorrhagic disease virus* (RHDV) in China in 1984 (Liu et al., 1984) led to the conclusion that it was a new and highly virulent viral disease of the European rabbit. The virus was presumed to have been introduced into China via a shipment of healthy German Angora rabbits (Cooke, 2002; Xu, 1991). Phylogenetic evidence demonstrates that the evolutionary origin of the Chinese lineage is European (Forrester et al., 2006a), although there had never been any substantiated reports of epidemic RHDV in Germany when the first recorded outbreak occurred in China in 1984. Thus, the virus must have been introduced as a persistent or an avirulent infection of the introduced healthy Angora rabbits. Retrospective evidence of RHDV-specific antibodies and RNA in healthy rabbits that lived before 1984 (Moss et al., 2002; Rodak et al., 1990) and the detection of a non-virulent strain of RHDV in domestic rabbits in Italy (Capucci et al., 1996) supported the hypothesis that RHDV might circulate silently amongst rabbits. Additionally, genomic-length RHDV-specific RNA was detected in healthy New Zealand rabbits exposed to virulent RHDV during controlled release experiments (Forrester et al., 2003; Zheng et al., 2002). We have therefore looked for direct evidence of RHDV persistence without disease. The present study describes the discovery of RHDV-specific antibodies in rabbits that colonise Lambay Island which is situated approximately 4 km off the Dublin coast of Eire. Importantly, there are no reports of rabbit haemorrhagic disease (RHD) on Lambay or in the Dublin area, the presumed source of the introduced rabbits. However, there is serological evidence of RHDV on Bull Island, nearby (Trout et al., 1997), and RHD epidemics have been reported in other parts of Ireland since 1995 (Collery et al., 1995; Forrester et al., 2006b). Our studies will show that a strain of RHDV has circulated innocuously for many years amongst an isolated wild rabbit population, possibly as a latent or persistent infection.

Results and discussion

Sera collected from 11 healthy rabbits were tested for the presence of RHDV-specific RNA using the primers and RT-PCR

☆ The sequence isolated in this work has been submitted to Genbank under accession no. DQ367359.

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sequencing as described in Materials and methods. Two samples produced cDNA molecules of the anticipated size (527 bp). This cDNA was purified and sequenced. The derived sequence data were assembled in a comparative alignment with other globally representative strains of RHDV (Table 1), and these alignments were used to construct a phylogenetic tree using PAUP* (Swofford, 2000) (Fig. 1). In addition to the relatively closely related strains of RHDV that are geographically widely dispersed, the highly divergent strains Ashington from the UK (Moss et al., 2002) and Rabbit calicivirus (RCV) from Italy (Capucci et al., 1996) were also included in the analysis. The tree (Fig. 1) shows that the viruses formed 8 groups in which groups 2–5 comprise European viruses, plus one strain of virus from Bahrain. However, groups 6–8 include the 1984 Chinese epidemic lineage as well as European, American, Middle Eastern, Australian and New Zealand virus strains of RHDV. The RNA sequence obtained from the sera of rabbits on Lambay Island was highly disparate from groups 2 to 8, diverging with Ashington (84.9% nucleotide identity) and RCV (81.0% nucleotide identity) in group 1. Moreover, the Lambay RNA sequence shared only 82.8% identity with the sequence of a typical European group 2 virus, i.e. the Frankfurt isolate. This is similar to the level of divergence between Ashington, RCV and the group 2 viruses. The tree topology also implies that the Ashington and Lambay Island lineages emerged after divergence of the lineage from RCV, possibly separating when the viruses were introduced into England and Ireland respectively. It is important to note that Ashington was isolated from a rabbit that died exhibiting the usual symptoms of RHDV, whereas RCV is known to be an avirulent virus that does not kill domestic rabbits (Capucci et al., 1996).

Table 1
Representative sequences aligned with the Lambay sequence providing an indication of the relationship of Lambay with other strains of RHDV

Sequence	Accession no.	Year of collection
Ashington	AF454050	1998
Ascot	AF454039	1992
Bahrain	DQ189077	2001
Bath	AF454024	2000
Bovey Tracey		1999
China 1984	AF402614	1984
China 1985	AY269825	1985
China CD	AY523410	
China tp	AF453761	
Czech V351	U54983	1988
Eisenhuttenstadt	Y15440	1989
Exminster0100	AF454010	2000
Exminster0300	AF454041	2000
France 88	U49726	1988
France 89	Z29514	1989
France 99	AJ302016	1999
France 00	AJ303106	2000
Frankfurt	Z15424	1996
Frensham		1999
Germany	NC_001543	
Hagenow	Y15441	1990
Hartmannsdorf	Y15425	1996
Horn Oak	AF454008	1999
Iowa	AF258618	2000
Ireland 1	AY925209	2001
Ireland 4	AY925210	2001
Ireland 12	AY926883	2001
Ireland 18	AY928269	2001
Ireland 25	AY928270	2001
Italy	X87607	1989
LogieAlmond	AF454016	1999
Maples Takely	AF454027	1999
Meiningen	Y15426	1992
Mellow Farm	AF454019	1999
Mexico	AF295785	1989
New Zealand	AF231353	
NZ (all samples)		2003
Park Farm 74	AF454047	1974
Park Farm 76	AF454048	1976
Perth		1999
Pennycombe Farm	AF454012	1999
Rainham	AJ006019	1993
Ramsey Island	AF454036	2000
RCV	X96868	1995
Sandscale Haws	AF454030	1999
Saudi Arabia	DQ189078	1996
Skomer		1999
Spain 89	Z49271	1989
Thetford	AF454044	1999
Triptis	Y15442	1996
Wellesbourne 55	AF454040	1955
Wellesbourne 58	AF454049	1958
Wellesbourne 59	AF454007	1959
Woking	AF454028	1999
Wriezen	Y15427	1996

In the phylogenetic tree (Fig. 1), the Lambay island lineage represents a significantly different virus from the other Irish viruses that were first described in 1995 (Nowotny et al., 1997) and have now been studied in more detail (Forrester et al., 2006b). Moreover, the Lambay lineage is apparently avirulent for rabbits but this has not been tested experimentally since no infectious virus has been isolated from the healthy rabbits on the island. The tree also shows that the virulent Irish strains in group 4 are closely related to other European strains and they emerged more recently than the Lambay lineage (see group 1 of Fig. 1).

ELISA antibody tests showed detectable antibody to RHDV in 10 of the 11 sera, with 9 having antibody titres greater than 1:1280, confirming that RHDV circulates efficiently and widely amongst the rabbits on Lambay Island without causing overt disease. In addition, 6 of the 11 samples contained virus-specific antibody to Myxoma virus (MYXV), with titres ranging from 1:20 to 1:320 demonstrating that MYXV co-circulates amongst the rabbits. The presence of high titre antibodies to both MYXV and RHDV is almost certainly due to circulating viruses, but as 8 of the sera tested were from young rabbits, the presence of residual maternally transferred antibodies cannot be excluded.

The most recent introduction of rabbits, presumably carrying RHDV, onto Lambay Island occurred approximately 20 years ago. This was before the identification of RHD and RHDV on the Irish mainland (Collery et al., 1995) or the identification of RHDV-seropositive wild rabbits in the Dublin area (Trout et al., 1997). The rabbit population density increased rapidly on Lambay Island despite the deliberate release of MYXV as a biocontrol agent, thus ruling out the possibility that the presence of the MYXV could have masked the presence of RHDV epidemics. Although there have been no systematic investigations, there are

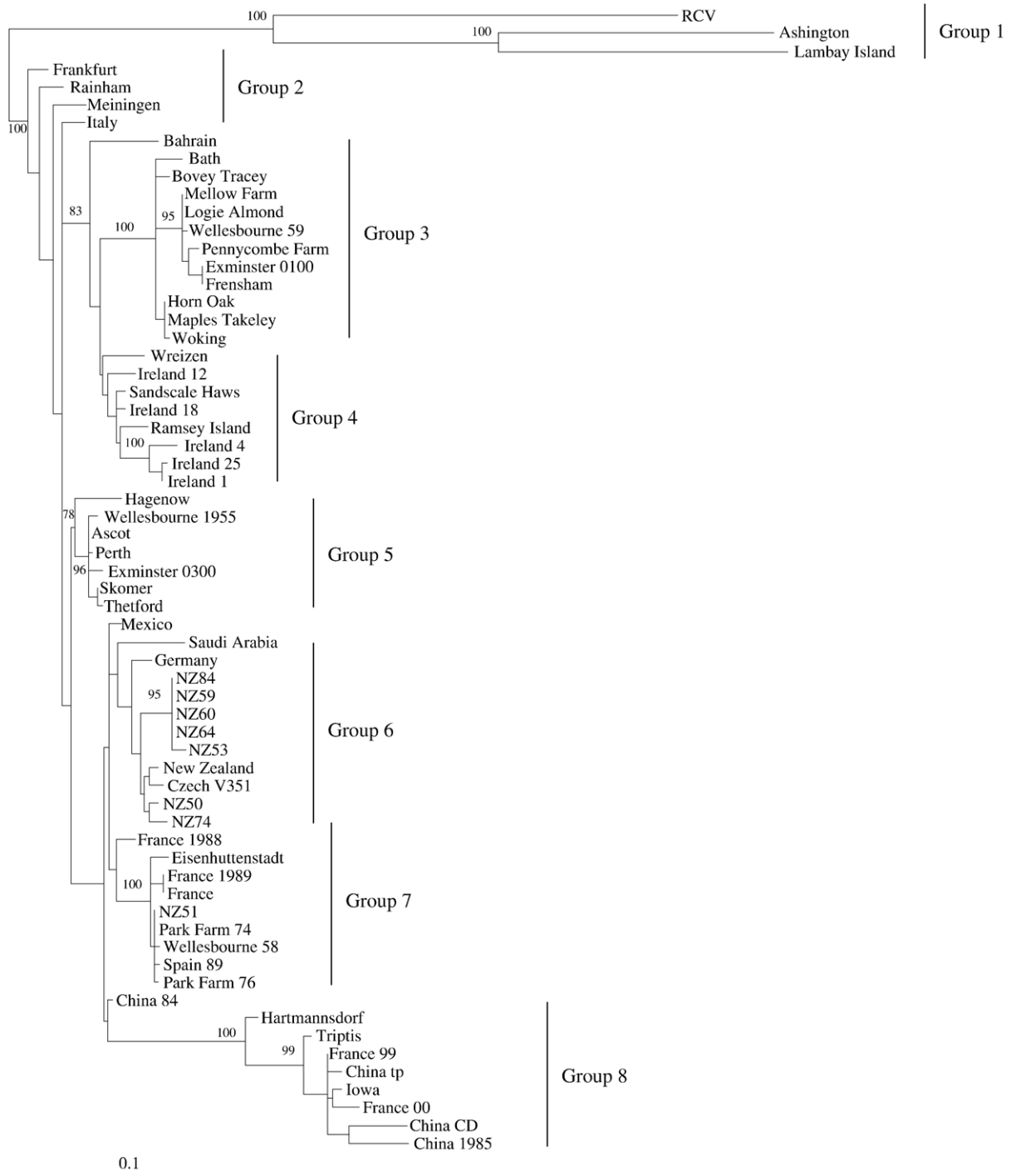

Fig. 1. Phylogenetic analysis using partial capsid sequence (see Materials and methods) for 64 strains of RHDV. Neighbour-Joining phylogeny was calculated using PAUP∗ version 4.0,10b. The optimal model to use with the data (HKY+Γ) was determined using MODELTEST 3.06, and the variable parameters were 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. The grouping of the viruses is intended to clarify the genetic relationships between the viruses following Forrester et al. (2006b). It does not necessarily identify virus subtypes.

no reports of epidemics due to RHDV on the island since the rabbits were introduced. Our results show that the rabbits on Lambay Island were seropositive for RHDV in the absence of overt disease. Moreover, in view of the rapid increase in rabbit population density and the isolation of these animals from potentially renewable sources of RHDV from the mainland, it seems unlikely that the virus has been sustained by unobserved virulent infections amongst the rabbits. Thus, the Lambay Island virus may have circulated in wild rabbits in the form of a persistent or latent infection. The alternative is that this virus shares with RCV the property of being an attenuated but infectious/contagious virus (Capucci et al., 1996). Whilst recognising the

limitations of virus sampling error, i.e. the small number of samples screened and the fact that most strains of RHDV have been isolated from dead rabbits, this is the third identification of a highly divergent RHDV lineage that appears to circulate only in a localised region, and in the case of at least two of these lineages, i.e. RCV and Lambay, they do not appear to circulate as virulent epidemic viruses of rabbits. We previously reported that, following the release of a highly virulent strain of RHDV in New Zealand, within a few years the livers of healthy rabbits were shown to contain genomic-length viral RNA corresponding to the virus released as the biocontrol agent, thus demonstrating that even lethal virus infects but does not necessarily kill rabbits (Forrester et al., 2003, 2006b). We believe this is compelling evidence that RHDV is able to cause persistent or latent infection in rabbits, and we hypothesise that, under appropriate circumstances, epidemic RHD outbreaks might arise, for example, by virus reactivation, perhaps under conditions of stress. However, this has never been observed for the attenuated variant RCV.

The discovery of a highly divergent RHDV lineage on Lambay Island suggests that the virus had circulated in Eire for many years before the typical European strain was identified in Ireland in 1995 (Collery et al., 1995). One can speculate that, although the Lambay strain has circulated within the Irish rabbit population for some time, it may have been outcompeted by the more recently introduced strain(s), only surviving in isolated pockets where there is no competition. It has previously been reported that antibodies against RHDV do not always protect against infection (Capucci et al., 1996, Marchandeau et al., 2005, Robinson et al., 2002). It is difficult to say whether or not this applies to the Lambay strain of RHDV, but the presence of antibodies in a very high proportion of healthy rabbits on the island suggests that the antibodies may be protective, otherwise one might expect to see periodic rabbit population fluctuations. It would be interesting to test whether or not rabbit populations from which the Lambay animals were derived on the mainland near Dublin are seropositive for RHDV. A similar situation could pertain with the Ashington strain in England which is also highly divergent and has only been identified once. From the phylogenetic tree, it is clear that the Lambay lineage emerged decades or even centuries before the extant strains shown in groups 2 to 5. It is possible that these divergent lineages represent viruses that have circulated for decades or even centuries, rarely causing sufficiently high fatality rates amongst rabbits to be observed and reported. It would be interesting to observe the consequence of housing a Lambay or Ashington rabbit with immunologically naive commercially supplied rabbits, or indeed challenging an antibody-positive rabbit from Lambay Island with a known virulent RHDV strain.

The presence of MYXV antibodies in the RHDV-seropositive rabbits on Lambay Island in the absence of myxomatosis suggests that either a low virulence strain of MYXV circulates on the island or that herd immunity on a small island was sufficient to protect the rabbits from MYXV. As far as we are aware, this is the first time that both RHDV and MYXV have been demonstrated to be co-circulating in a rabbit population in the absence of overt disease. There is unpublished evidence that MYXV has recently caused an epidemic of myxomatosis on Lambay (R. Trout, personal communication), whether or not this will impact on the epidemicity of RHDV remains to be seen.

The discovery of the Lambay Island RHDV lineage provides convincing evidence that this virus can survive long-term in wild rabbit populations without causing overt disease. Whilst we have previously demonstrated that virulent strains of RHDV can establish innocuous infections in rabbits (Forrester et al., 2003), it has not been formally proven that this was a persistently circulating strain of RHDV. The presence of antibodies in the rabbits on Lambay Island, with no evidence of disease for decades, is compelling evidence that RHDV has been circulating either as a persistent/latent infection or as an avirulent form of the virus in wild rabbits. Presumably, this virus also circulates silently at least in the Dublin area of Eire, i.e. the geographic source of the rabbits in the 1980s. Clearly, further studies are needed to understand more fully the epidemiology, maintenance and pathogenetic determinants of RHDV within rabbit populations.

Materials and methods

Samples of serum separated from blood cells were collected from apparently healthy rabbits killed on Lambay Island (latitude: 53° 29′.30 N, longitude: 06° 02′.46 W). The history of this Island dates back to the Mesolithic period (circa 7000 years BC). Archeological and documented evidence suggests that it has been continuously inhabited and today it remains a popular area for scuba divers, fishermen and ornithologists. During early 1959, the rabbit densities were very high, resulting in adverse affects on the flora and fauna of the Island. Consequently, Myxoma virus (MYXV) was deliberately introduced as a biocontrol agent and the rabbit population was rapidly reduced by myxomatosis. The additional impact of predation and other factors eradicated them. Subsequently, in 1985, healthy rabbits were re-introduced onto the island from the Dublin area of Eire. As far as can be determined, rabbits were only re-introduced once (M. Kelly, personal communication). Since their re-introduction, these rabbits have progressively increased in numbers, reaching extremely high population densities during the last few years. Attempts to reduce the rabbit densities were made by the re-introduction of MYXV, but the virus had little effect on rabbit numbers.

RT-PCR was performed as described in Moss et al. (2002). The resulting sequences were aligned with published representative RHDV sequences. 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 and Crandall, 1998). The optimal model (HKY + Γ) was then used to estimate the Maximum Likelihood tree using iterative heuristic 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. Neighbour-Joining bootstrap support (1000 replicates) was calculated for each tree using the Maximum Likelihood model.

ELISA tests for the detection of antibodies to RHDV were carried out on the serum as described in Moss et al. (2002).

ELISA tests for the detection of antibodies to Myxoma virus were carried using purified MYXV as described in Gelfi et al. (1999), and the ELISA protocol as described by Moss et al. (2002) was subsequently followed.

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