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Short communication

Evolution of rabbit haemorrhagic disease virus (RHDV) in the European rabbit (*Oryctolagus cuniculus*) from the Iberian Peninsula

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ABSTRACT

To date information on rabbit haemorrhagic disease virus (RHDV) in Spain and Portugal has been scarce, although the disease is endemic and continues to have a considerable impact on species conservation and hunting industry. We analysed RHDVs obtained between 1994 and 2007 at different geographic locations in Portugal (40 samples), Spain (3 samples) and France (4 samples) from wild European rabbits (*Oryctolagus cuniculus*) that succumbed to the disease. Phylogenetic analyses based on partial VP60 gene sequences allowed a grouping of these RHDVs into three groups, termed "Iberian" Groups IB1, IB2 and IB3. Interestingly, these three Iberian groups clustered separately, though not far from earlier RHDVs of Genogroup 1 (containing e.g., strain "AST89"), but clearly distinct from globally described RHDV strains of Genogroups 2–6. This result, supported by a bootstrap value of 76%, gives rise to the hypothesis that the virus evolved independently since its introduction to wild rabbit populations on the Iberian Peninsula, with the Pyrenees acting as a natural barrier to rabbit and hence to virus dispersal. No differences were observed in RHDV sequences obtained from geographic regions where the rabbit subspecies *O. c. algirus* prevails compared with those obtained from *O. c. cuniculus*.

1. Introduction

Rabbit haemorrhagic disease (RHD) outbreaks on the Iberian Peninsula were first described in 1988 and the disease is endemic since (Anon, 1989; Arguello Villares et al., 1988). Wild rabbit abundance has declined over 30% since, raising conservational concerns and having a negative economic impact on the hunting industry (Alves and Ferreira, 2004; Moreno et al., 2007; Villafuerte et al., 1995). The European rabbit that nowadays is distributed in many continents originated from the Iberian Peninsula, where two well separated subspecies exist, *Oryctolagus cuniculus cuniculus* in the northeast and *Oryctolagus cuniculus algirus* in the southwest, which form a contact zone in the central region (Branco et al., 2000; Geraldes et al., 2006; Monnerot et al., 1994) (Fig. 1). The subspecies *O. c. algirus* is endangered due to various factors, but, most importantly, due to high mortality rates of RHD epizootics (Alves and Ferreira, 2004; Moreno et al., 2007).

Surprisingly, the available genetic information on rabbit haemorrhagic disease virus (RHDV) in Spain and

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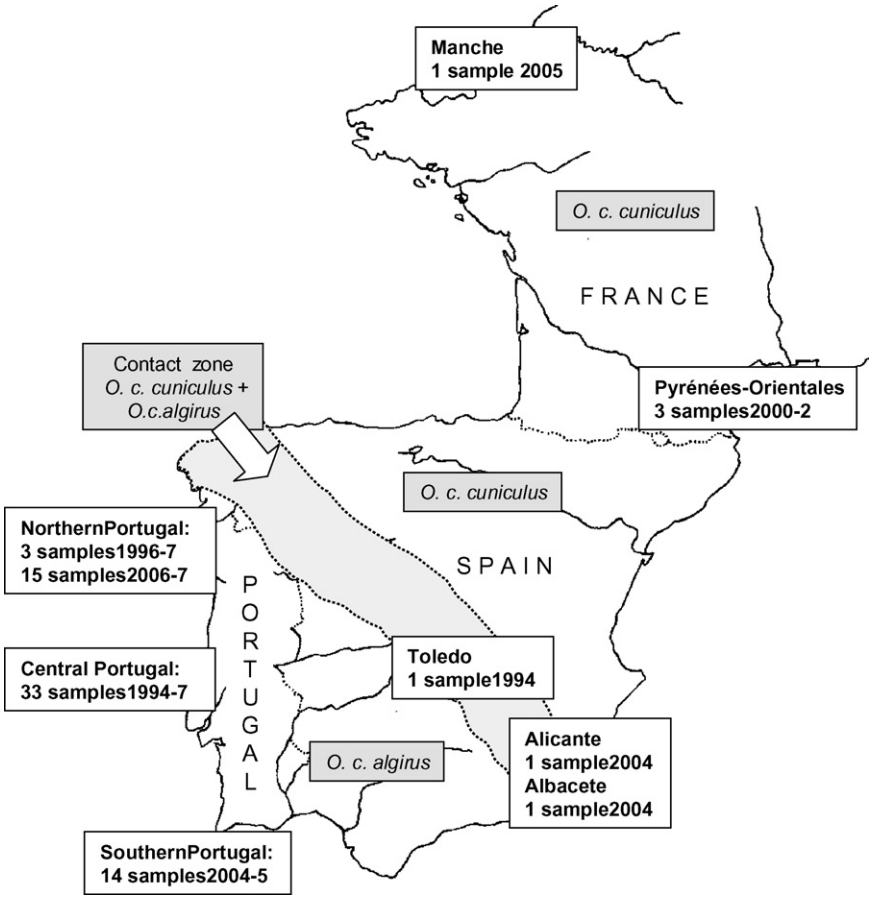

Fig. 1. Map of the Iberian Peninsula and South of France displaying the geographic origin of the RHDV samples analysed in this study and the time period they were collected. The distribution areas of the wild rabbit subspecies *Oryctolagus cuniculus algirus* and *Oryctolagus cuniculus cuniculus* as well as the contact zone across the Iberian Peninsula are indicated.

Portugal is scarce (Boga et al., 1994; Nowotny et al., 1997; Parra and Prieto, 1990), although many studies have been conducted in other countries (e.g. Forrester et al., 2007; Le Gall-Reculé et al., 2003; Le Gall et al., 1998; Matiz et al., 2006; Moss et al., 2002; Nowotny et al., 1997). These phylogenetic analyses focus predominantly on sequences located on the viral capsid protein VP60 gene, and although inferences could be made in some studies, linking RHDV genetic groups to variables such as the years of sampling, geographic origin, or virulence of strains, these relations were not always observed and therefore cannot be generalized.

Our objectives were to characterise RHDV strains obtained from wild rabbits presumed to have died from RHD in different years and geographic locations in the Iberian Peninsula as well as to characterise RHDV strains obtained from rabbits in distribution areas of *O. c. algirus*.

2. Materials and methods

2.1. Samples

In Portugal, a total of 65 wild rabbits that were found dead during known epidemics of RHD were collected and immediately stored at −20 °C. The animals were collected from different geographic areas in different years (Fig. 1). Thirty-six samples were collected between 1994 and 1997 for a study aiming to determine the presence of RHDV as cause of death in wild rabbits. These had previously tested positive by antigen capture ELISA (Capucci et al., 1991). These and the remaining liver samples (collected 2004–2007) were processed in the Laboratory of the University of Porto.

Seven RHDV sequences were obtained from wild rabbits from Spain and France (Table 1; Fig. 1). The sequence from the Toledo specimen was obtained in the Laboratory of the University of Porto. It was the only nested RT-PCR positive sample out of 39 hunted wild rabbits collected in 1994. The remaining RHDV sequences from wild rabbits from Spain and France were obtained in the Laboratory of AFSSA in Ploufragan. These refer to the two Spanish RHDVs collected in Alicante and Albacete in 2004 kindly provided by Dr. Ramon Soriguer (CSIC) and to the four recent RDHV sequences from France "2000–08", "2001–23" and "2002–20" that had been collected in 2000, 2001 and 2002, respectively, in the Department "Pyrénées-Orientales", and to "2005–01" that was obtained in 2005 in the Department "Manche".

Table 1
Genbank accession numbers of RHDV sequences included in the phylogenetic analysis.

RHDV group	Strain	GenBank reference
Group IB3	2004–03	EU192134
	France_2001–23	AM746980
	Spain_Alicante_2004	AM884394
	Spain_Albacete_2004	AM884395
	2006–01	EF571322
	2006–04	EF571325
	2006–09	EF571330
	2005–01	EU192140
	France_2000–08	AJ319594
	France_2002–20	AM746981
	2007–01	EU192135
Group IB2	1995–01	EU192132
	1994–02	EU192136
	1997–03	EU192139
	Spain_Toledo_1994	EU192137
	1996–08	EU192138
Group IB1	1997–02	EU192133
	1994–07	EU192131
Genogroup 1	Eisenhuettenstadt	Y15440
	AST89	Z49271
	France_SD	Z29514
	Spain_MC-89	L48547
Genogroup 2	China_WX/1984	AF402614
	Mexico89_	AF295785
	Germany_FRG	M67473
	Czech_V351	U54983
Genogroups 3-5	France_00–13	AJ495856
	France_2005–01	AM085133
	Wriezen	Y15427
	Hagenow	Y15441
	Meiningen	Y15426
	Frankfurt	Y15424
	France_95–10	AJ535094
Genogroup 6	CUB5-04	DQ841708
	France_03–24	AJ969628
	France_99–05	AJ302016
Root	RCV	X96868

2.2. RT-PCR and sequencing

RNA was extracted from liver homogenates and cDNA was synthesized using random priming and M-MLV reverse transcriptase (Invitrogen). Nested PCR was used to amplify partially the RHDV VP60 capsid protein gene (Moss et al., 2002), corresponding to positions 6157–6703 of strain AST89 (GenBank accession number Z49271). The amplicons of 547 bp were directly sequenced in both directions employing the nested PCR forward and reverse primers.

2.3. Sequence analysis

From Portugal only the 40 non-identical sequences were further analysed. For phylogenetic analysis, different methods (minimum evolution, maximum parsimony, maximum likelihood and neighbour-joining) were applied using the software package MEGA3.1 (Kumar et al., 2004). The obtained trees showed similar clustering of the sequences. The neighbor-joining tree was presented as considered adequate for comparing relatively short sequences (Takahashi and Nei, 2000). The nucleotide substitution model of Kimura-2-parameter was used, and the reliability of the tree was tested by bootstrap analysis of 1000 replicates. The obtained sequences were compared with published homologues from GenBank database (NCBI). Eighteen sequences representing previously described RHDV genogroups were selected and included (Table 1). These sequences were grouped into Genogroup 1–6, adapted to the classification used by Le Gall-Reculé et al. (2003). The Italian non-pathogenic rabbit calicivirus "RCV" (GenBank accession number X96868) was used to root the tree. The amino acid sequences and the consensus sequences of each group were deduced and aligned.

3. Results

The phylogenetic tree estimated by the neighbor-joining method (Fig. 2) shows the clusters formed by RHDV strains from the Iberian Peninsula and the south of France, which have been termed "Iberian" Groups IB1–IB3, together with the clusters containing "known" RHDV strains representing Genogroup 1, Genogroup 2, Genogroups 3–5 and Genogroup 6 antigenic variants or "RHDVa" strains (Capucci et al., 1998). The Groups IB1 and IB2 contain sequences collected between 1994 and 1997, whereas Group IB3 includes more the ones collected between 2000 and 2007. Each group contains sequences from different geographic locations, except Group IB1, that contains five sequences obtained in 1994 and 1997 from Santarém district of central Portugal. Group IB2 contains strains collected between 1994 and 1997 from northern and central Portugal as well as the virus obtained in 1994 from Toledo, Spain. Group IB3 includes the more recent (2004–2007) Portuguese strains collected in the southern province Algarve (2004–2005) as well as in the North (2006–2007). Also included in Group IB3 are the sequences from three French RHDVs that had been identified between 2000 and 2002 in the Department "Pyrénée-Orientales", and the two Spanish strains "Albacete" and "Alicante" collected in 2004.

All described RHDV sequences from Portugal, Spain and the South of France represented in Groups IB1, IB2 and IB3 formed, together with Genogroup 1 sequences, a distinct cluster separated from all previously described RHDV genogroups, supported by a bootstrap value of 76%. Groups IB2 and IB3 viruses share a common ancestor with Genogroup 1 viruses (bootstrap 82%). Group IB1, which contains five sequences from central Portugal (bootstrap 97%), separated before the known RHDV Genogroup 1. The recent French strain "2005–01" obtained in the Department "Manche" clustered within Genogroups 3–5, close to another French strain collected in 2000 ("00–13").

The grouping of the RHDVs was supported by the bootstrap values as well as a comparison of nucleotide proximities. Nucleotide similarities of 97%, 98% (excluding strain "1996–08") and 99–94% were observed within Groups IB1, IB2 and IB3, respectively. Lower values were observed between groups and genogroups. Group IB2 strain "1996–08" displayed a higher genetic distance proportionally to all other strains, but was still closer to RHDVs of its

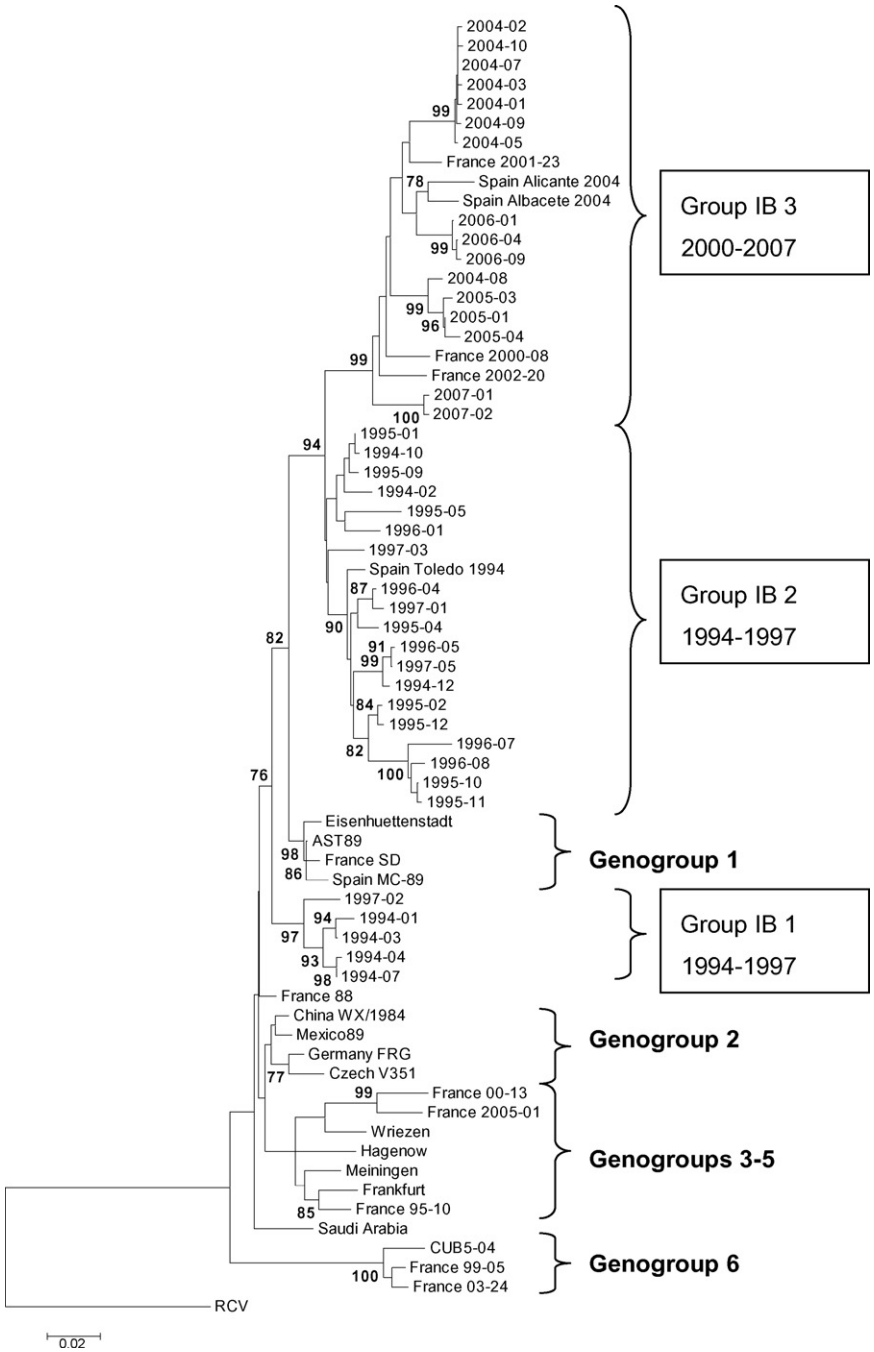

Fig. 2. RHDV strains from Portugal cluster separately from known genogroups based on phylogenetic analysis of partial VP60 gene sequences. The neighbour joining tree was rooted with RCV. Bootstrap probability values above 75% for 1000 replicate runs are indicated at the nodes.

own Group IB2 (96–95%) than to Genogroup 1 strains (94%) or others (less than 94%).

The comparison of the group consensus sequences of the deduced RHDV VP60 polymorphic amino acid composition showed that, in comparison to Genogroup 1, Group IB1 presents three replacements: $A_{2176}N$, $N_{2178}S$ and $S_{2194}N$. The strains contained in Groups IB2 and 3 are characterised by five amino acid replacements when the respective group consensus sequences are compared to those of Genogroups 1–6: $R_{2062}Q$, $L_{2152}P$, $T_{2163}I$, $A_{2176}T$ and $V_{2230}I$. The more recent Iberian and French RHDV strains of Group IB3 are further characterised by the residues $P_{2069}N$, $N_{2071}G$ and $I_{2092}V$, which is not a feature of the other described strains.

4. Discussion

In Spain and Portugal, rabbit mass mortality due to RHD was observed in the late 1980s and early 1990s,

coinciding with the observation of the epidemic in other European countries, suggesting that the origin of the virus was the same. Accordingly, Genogroup 1 sequences were obtained from RHD outbreaks in 1989 not only in Spain (Boga et al., 1994; Parra and Prieto, 1990), but also in France (''France SD'') and Germany (''Eisenhuettenstadt''). No genetic information has ever been published on more recent RHDVs circulating in wild rabbits in Spain, and none ever from Portugal. Here, 47 RHDV strains from wild rabbits (*O. cuniculus*) obtained between 1994 and 2007 at different geographic locations in Portugal, Spain and France (Fig. 1) were characterised based on their partial VP60 gene sequences and grouped into three groups, termed ''Iberian'' Groups IB1, IB2 and IB3. All these ''Iberian'' Groups sequences formed, together with those of Genogroup 1, a distinct cluster separated from all other described RHDV genogroups. Group IB2 and IB3 viruses share a common ancestor with Genogroup 1 viruses, whereas Group IB1 strains separated before Genogroup 1 RHDV.

In geographically neighbouring France, virulent RHDVs of two distinct Genotypes (1 and 2) were initially present between 1987 and 1990, but were subsequently replaced by other RHDV strains (Le Gall-Reculé et al., 2003). Despite the lack of data between 1989 and 1994, none of the RHDV sequences from Spain and Portugal clustered within Genogroup 2, suggesting that only Genogroup 1-like virulent RHDV strains predominated initially and that these were subsequently replaced by strains here grouped as IB2 and IB3, indicating that RHDV could have evolved separately in the Iberian Peninsula since. Alongside the nucleotide sequence analysis, the observed amino acid polymorphisms suggest that the substitutions observed in Groups IB2 and IB3 seem to have become fixed around 1994 and are still present in RHDVs circulating in wild rabbits in the Iberian Peninsula, but not elsewhere. It is therefore tempting to speculate that the Pyrenees may act as a natural barrier, constraining wild rabbit and hence virus dispersal and evolution, similar to what was observed on Lambay island or in New Zealand (Forrester et al., 2007; Forrester et al., 2003).

Phylogenetic, serological and epidemiological studies related to RHD have led to the hypothesis that attenuated or avirulent forms of the virus have been circulating in Europe before the 1980s (Forrester et al., 2003; Moss et al., 2002; Nowotny et al., 1992; Rodák et al., 1990). The drastic reduction in wild rabbit numbers observed on the Iberian Peninsula has been historically unprecedented, suggesting that, if any RHDV-like viruses were circulating in wild rabbit populations at that time, they must have been highly host-adapted but not cross-protective. Among the 39 wild rabbits obtained from hunters in 1994 from the Toledo region, only one tested positive by nested RT-PCR (2–3% of the total). Also, more recently in 2006, liver samples obtained from 30 healthy wild rabbits hunted in 2006 in Pancas (Lisbon district) tested negative by nested RT-PCR (data not shown). This data is different from the high percentage (40–60%) of PCR positive healthy wild rabbits found in New Zealand (Forrester et al., 2003), suggesting that in the Iberian Peninsula RHDV-like viruses are either not present or are present at a very low incidence. Climatic differences together with potentially circulating avirulent ''RHDV-like'' strains in cooler and more humid countries (Cooke, 2002) may explain the observed differences.

The categorisation of the samples obtained in Portugal between 1994 and 1997 in two distinct groups (IB1 and IB2) was quite surprising, as all were collected during the same time period, and because the five RHDVs that formed Group IB1 originated from the same district (Santarém) as others that grouped within IB2 (RHDV strains ''1994–10'', ''1995–01'', ''1995–10'', ''1995–11''), suggesting that two different RHDVs were circulating concomitantly in the presence of disease and mortality. The alignment of the amino acid sequence group consensus sequences further strengthens this hypothesis, as Group IB1 differed in eight positions when compared to Group IB2, but in only three when compared to Genogroup 1. Bearing in mind, that RHDV infection can perpetuate in rabbit holdings due to virus persistence (Gall et al., 2007; Moss et al., 2002), i.e. without repeated introduction of the virus from the environment, the question arises whether the detected subgroups are typical for wild rabbits of this region. Our data seem to support this hypothesis, as e.g., shown by the proximity of all recent wild rabbit RHDVs collected on the Iberian Peninsula and South of France forming Group IB3. This does not necessarily mean that the same RHDV circulate in wild and farmed or pet rabbits. We characterised a RHDV from an outbreak that occurred in January 2007 in a commercial rabbit farm in the North of Portugal, which was classified as an antigenic variant RHDVa, based on partial VP60 gene sequence analysis (own observations) and on antigenic characterisation using a panel of monoclonal antibodies performed by the OIE Reference Laboratory (Dr. L. Capucci).

Phylogenetic analyses of RHDV strains are commonly based on sequences representing only a fragment of the capsid VP60 protein gene, however, discrepancies between authors in relation to the number of RHDV groups and subgroups and also in tree topology, warrant harmonisation of RHDV typing. Due to recombination events contributing to RHDV variability, it may be more appropriate to investigate the complete sequence encoding VP60, or ideally, the full length genome, rather than partial capsid gene sequences (Abrantes et al., 2008; Forrester et al., 2008).

In this study we report the genetic characterisation of RHDV strains which have been obtained in Portugal, where the rabbit subspecies *O. c. algirus* is prevalent, from locations in Spain within the contact zone, and from France where only *O. c. cuniculus* has been described. No significant epidemiological, clinical or pathological differences have been observed between the rabbit subspecies, suggesting that *O. c. algirus* is as susceptible to RHDV as *O. c. cuniculus*, and that the virus is equally virulent for both rabbit subspecies. No significant differences have been observed in RHDV partial VP60 gene sequences obtained from wild rabbit specimens in either region. To our knowledge this is the first genetic characterisation and molecular epidemiology of RHDV sequences obtained from *O. c. algirus*.

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