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Short communication

Detection of rabbit haemorrhagic disease virus (RHDV) in nonspecific vertebrate hosts sympatric to the European wild rabbit (*Oryctolagus cuniculus*)

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ABSTRACT

Since its detection in China in 1984, rabbit haemorrhagic disease (RHD) has been the subject of numerous studies. Yet, the evolutionary origin of rabbit haemorrhagic disease virus (RHDV) is still under debate. For example, some aspects related to the epidemiology of the disease are still unknown, such as where the virus is hosted between RHD outbreaks. To detect the presence of RHDV in rabbit-sympatric micromammals, 51 rodents (29 *Mus spretus* and 22 *Apodemus sylvaticus*) and 31 rabbits (*Oryctolagus cuniculus*) from the same location in central Spain were analyzed. In those samples in which the virus was detected, a fragment of the VP60 protein gene from the RHDV capsid was sequenced and the phylogenetic relationships between them and other strains of RHDV in the Iberian Peninsula were analyzed. In total, five viral strains were identified in *A. sylvaticus*, *M. spretus* and *O. cuniculus*. All strains were found to be well supported within the clade of RHDV found in rabbits in the Iberian Peninsula. Moreover, one of the strains was found in all three species under study, which suggests the capability of RHDV to infect other mammals apart from the rabbit which have not yet been investigated. The transmission of the virus is discussed as well as its ecoepidemiological implications.
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1. Introduction

Rabbit haemorrhagic disease (RHD), first discovered in China in 1984 (Liu et al., 1984), has a high rate of morbidity (100%) and mortality (40–100%) in adult European wild rabbits (Henning et al., 2005). Following its discovery in Spain in 1989, many populations of European wild rabbit have suffered high mortality rates of close to 80% (Villafuerte et al., 1994). Since then, rabbit numbers have declined at a relatively constant rate (Moreno et al., 2007). RHD is caused by a positive-sense, single-stranded RNA virus, a member of the genus *Lagovirus* within the family Caliciviridae (Parra and Prieto, 1990). This genus also includes the European brown hare syndrome virus (EBHSV), as well as the non-pathogenic rabbit calicivirus (RCV), which causes asymptomatic seroconversion in rabbits and is considered a potential apathonic ancestor of RHDV (Capucci et al., 1996). Recently a new calicivirus has been described, named the Michigan rabbit calicivirus (MRCV), which causes subclinical infections and whose genome shows an average similarity of 79% with RHDV (Bergin et al., 2009). However, further studies have questioned this finding and have proposed instead that MRCV is not a novel calicivirus but a new variant of the nonpathogenic RCV-like group (Abrantes and Esteves, 2010).

Since its identification, RHDV has been the subject of numerous studies. Yet, some aspects, such as the evolutionary origin of the virus, are still unknown or under debate (Forrester et al., 2006; Kerr et al., 2009). Various hypotheses have been put forward to explain the origin of RHDV, such as (i) the transference of EBHSV from the European brown hare (*Lepus europaeus*) to the rabbit (Nowotny et al., 1997), (ii) that changes were produced in a non-pathogenic virus, rendering it virulent (Moss et al., 2002), or (iii) the emergence of RHDV from another virus infecting another species (Fenner and Fantini, 1999).

All lagoviruses, with the exception of EBHSV, which solely affects hares (Löliger and Eskens, 1991), are specific to *Oryctolagus cuniculus* in domestic and wild forms, although the latter prove

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more sensitive to the virus (Argüello et al., 1988; Pagés, 1989). Despite the fact that RHDV and EBHSV show a genetic similarity of 71% and are also related antigenically, as shown by the presence of common internal epitopes (Wirblich et al., 1995), cross-infection does not occur (Lavazza et al., 1996). Equally, experimental infection of diverse species of laboratory and wild animals with RHDV has produced no symptoms of the disease (Gavier-Widen et al., 1997; Nowotny et al., 1999; Smid et al., 1991). Nor have other species of American leporids (eastern cottontail *Sylvilagus floridanus*, volcano rabbit *Romerolagus diazi*, and black-tailed jackrabbit *Lepus californicus*) been clinically affected upon experimental exposure to RHDV (Gregg et al., 1991). Likewise, non-target mammals during periods of viral activity in New Zealand were not affected (Parkes et al., 2004). The replication of RHDV in other hosts apart from the rabbit has not been established. In Australia, seroconversion has been detected as a response to the viral antigene in mice inoculated with the virus (Gould et al., 1997) as well as in kiwis (*Apteryx australis*) exposed to contaminated material (Buddle et al., 1997). Similarly, in red foxes (*Vulpes vulpes*) that had ingested rabbits infected with RHDV, post-infection antibody titers were detected, without the viral replication ever fully manifesting (Leighton et al., 1995).

The main natural transmission mechanism of RHDV is direct contact between infected rabbits through respiratory and oral routes and skin lesions (Xu et al., 1988). Subclinically ill or infected rabbits are the principal sources of infection (Xu and Chen, 1989). Moreover, rabbits with persistent infection (Löliger and Eskens, 1991), carriers (Cancellotti and Renzi, 1991; Cooke, 2002), as well as young rabbits under 20 days old who manage to survive the disease (Rosell et al., 1989), can also act as reservoirs of the virus. However, possible alternative hosts of the virus and their role in the epidemiology of the disease have scarcely been studied. Some field and laboratory studies have shown how predatory mammals and birds play a role in the transmission of RHDV (Chasey, 1994; Gavier-Widen and Morner, 1993; Simón et al., 1994), acting as mechanical reservoirs or vectors. Schirrmeier et al. (1990) conducted an experiment with rodents and insects, suggesting that these did not act as reservoirs; they did, however, act as possible passive transmitters of the virus. Likewise, other studies prove the importance of the role played by insects in transmission (Asgari et al., 1998; Gehrmann and Kretzschmar, 1991; Lenghaus et al., 1994), as well as that of the decomposing remains of the infected rabbits themselves (McColl et al., 2002), or their warrens in housing the RHDV (Calvete et al., 2002).

In this paper, the possible presence of RHDV is analyzed in two species of rodent (wood mouse *Apodemus sylvaticus* and Algerian mouse *Mus spretus*) and in the European wild rabbit, which all share the same habitat in a specific area of central Iberian Peninsula. This association is vital to extend knowledge on the role that other wild species (which have not yet been investigated) may play both in the origin and in the epidemiology of this disease.

2. Material and methods

2.1. Study area and sampling methods

The study area is situated in the municipality of Plasenzuela in Extremadura, Spain (39°22′N 6°02′W). It is classified as a high rabbit relative abundance area (Blanco and Villafuerte, 1993), with a hunting bag of 780 rabbits per year (Junta Extremadura, 2005; pers. comm.). The land is a rich thicket of mostly *Retama sphaerocarpa* shrubs, interspersed with rocks and occasional holm oak trees (*Quercus ilex*). The climate is typically Mediterranean; mild winters and very hot summers of variable temperature and precipitation (average of <500 mm per annum).

Rodents were captured alive, from 5th July to 25th July 2005, using wire mesh traps ("Hipólito" traps) (Carro et al., 2007). A trapping grid ($N = 10$) covering 5 ha was set up within the study area. Fried bread was used as bait. Wire mesh traps were placed at the entrance or in the vicinity of active rabbit warrens (<1 m from the entrance). Rows of ten traps per hectare, spaced up to 20 m apart, were set at night and checked each morning. The capture effort was maintained for 20 nights and traps were not relocated until a minimum of 10 micromammals per hectare was reached. In total, 51 micromammals (29 Algerian mice, *M. spretus*, Ms1–Ms29 and 22 wood mice, *A. sylvaticus,* As1–As22) were captured. No other species was captured. Each mouse captured was sacrificed "in situ" by cervical dislocation. Each animal was placed in an individual bag, properly identified and refrigerated for transportation to the laboratory, where they were frozen at −20 °C.

Additionally, 31 rabbits (*O. cuniculus*, Oc1–Oc31) were shot by hunters during an exceptional legal hunting period made available from the 15th of July to the 15th of August 2005 in the same area of study. Samples were handled and stored as described above for mice.

2.2. Laboratory methods

The RHDV sampling consisted in the extraction of the entire or parts of the liver from the animals captured. This task was carried out at different times and using sterile laboratory material, under strict biosecurity measures to avoid any cross-contamination. Furthermore, and to the same end, the different species (*O. cuniculus*, *A. sylvaticus* and *M. spretus*) were treated in separate laboratories. All samples were processed on premises belonging to the Caceres Veterinary School of the University of Extremadura (Spain).

Viral RNA was extracted from an organ homogenate of 0.1 g in 1 ml phosphate buffered saline (PBS) using TRI® Reagent LS (Sigma). cDNA was synthesized using random priming and M-MLV reverse transcriptase (Invitrogen). To test for positive samples, nested PCR was used to partially amplify RHDV capsid protein gene VP60 (Moss et al., 2002). The reaction mixture (25 μl), for PCR I and PCR II, contained 2.5 μl of 10× PCR buffer, 0.6 μl of each primer (10 mM), 100 μM of each dNTP, 2.0 μl $MgCl_2$ (25 mM), 1 U Taq DNA polymerase (Fermentas) and 1 μl of cDNA or product from first PCR. Primers RHDV1 and 4 were used for the first PCR and primers RHDV2 and 3 for the nested reaction. For both PCRs the cycling conditions were 30 cycles of 95 °C for 40 s, 50 °C for 40 s, 72 °C for 2 min and a final elongation of 72 °C for 10 min, in a thermocycler C 1000 (Bio-Rad). The size of the obtained PCR products was determined by ethidium bromide staining of a 2% agarose gel electrophoresis. The expected size of the final PCR product was 573 bp corresponding to the region covering positions 6151–6724 of the major capsid protein VP60 of RHDV of the Spanish RHDV strain AST89 (Z49271). The molecular analyses were carried out in the Clinical Veterinary Practice Department laboratories of the Abel Salazar Biomedical Sciences Institute (ICBAS), University of Porto (Portugal). PCR amplicons were purified and sequenced in both directions, in an ABI 3730XL (Applied Biosystems) using BigDye® terminator kit v3.1 (Applied Biosystems) with primers RHDV2 and 3. These analyses were performed by STAB VIDA (Portugal). All sequences obtained were deposited in Genbank with the following access numbers: HQ198365–HQ198366, HQ198368–HQ198371, HQ413340–HQ413341.

2.3. Phylogenetic analysis of RHDV VP60

Chromatograms were checked and assembled using Sequencher 4.6 (Gene Codes Corporation). Consensus nucleotides were translated into amino acids using MacClade 4.05 (Maddison

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and Maddison, 2000) and manually aligned with other homologous sequences available in GenBank from the Iberian Peninsula (Alda et al., 2010; Müller et al., 2009) and representatives from RHDV Genogroups 1–6 previously described (Le Gall-Recoulé et al., 2003). Rabbit calicivirus (RCV) (X96868) was used as an outgroup.

In order to identify the most appropriate evolutionary model by the Akaike corrected information criterion (AICc), the program jModeltest 0.1.1 was used (Posada, 2008). Phylogenetic relationships among all the RHDV strains were inferred using Bayesian Inference (BI) in MrBayes 3.1.2 (Ronquist and Huelsenbeck, 2003), simulating four simultaneous Markov chains (MCMC) for 4×10^6 generations each, and using a sampling frequency of 100 generations. Convergence between run parameters from paired simultaneous runs was adjusted considering an adequate sampling based on average standard deviation of split frequencies being <0.01 (Huelsenbeck and Ronquist, 2005). The program Tracer v1.5 (Rambaut and Drummond, 2007) was used to determine burn-in length considering the set of trees saved prior to log likelihood stabilization and convergence.

3. Results

3.1. Detection of RHDV in mice and rabbits

All sampled rodents appeared healthy. RHDV positivity was detected by molecular analysis in the liver of 3 mice (5.8%): 2 *A. sylvaticus* (As15 and As26) and 1 *M. spretus* (Ms8). The rabbits analyzed also appeared healthy, showing no sign of the disease. Out of the 31 rabbits caught, the virus was detected in 7 (23.5%), of which 6 provided analysable sequences (Oc1–Oc6).

3.2. Phylogenetic relationships of RHDV isolated in rabbits and mice

Along with the homologous sequences available in GenBank from the Iberian Peninsula and representative from the main RHDV lineages, we constructed an alignment of 82 sequences. The best evolutionary model estimated by jModelTest 0.1.1 for our data was TVM + G, and the gamma shape parameter was $G = 0.366$.

In all of the animals analyzed, 5 strains of virus were found, according to the sequences obtained from the VP60 gene. The phylogenetic analysis performed was congruent with previous studies, and indicated that all Iberian samples form a monophyletic group (Alda et al., 2010; Müller et al., 2009). All of the virus strains detected in this study were found within this Iberian lineage (Genogroup 1, Fig. 1). Three rabbits (Oc1, Oc3 and Oc6), one *A. sylvaticus* (As15) and one *M. spretus* (Ms8) showed the same sequence of RHDV VP60 capsid gene, identical to a strain identified in Portugal in 2004 (EU192134, Müller et al., 2009). This strain was very similar to those detected in two other rabbits (Oc4 and Oc5), which differed one nucleotide from each other (*p*-distance = 0.002), and they came together to form a highly supported clade, which was the sister group of another series of strains isolated in Spain between 2004 and 2006 (Fig. 1). The other two identified strains of RHDV, corresponding to one rabbit (Oc2) and one *A. sylvaticus* (As26) grouped together (*p*-distance = 0.028) and formed a differentiated and supported clade containing strains isolated at a much earlier point in time, i.e. between 1994 and 2005 in Spain and Portugal (Fig. 1).

4. Discussion

To our knowledge, this is the first time that the natural presence of RHDV in wild micromammals *A. sylvaticus* and *M. spretus* has been detected. These species were captured in the immediate vicinity of warrens in areas of high relative abundance of rabbits. Unfortunately, the reduced sample size in this study does not allow to establish significant differences regarding infection rates of RHDV in *A. sylvaticus* (9%) and *M. spretus* (3.4%). Nevertheless, we might hypothesize that infection rates most likely depend on the abundance of each species in the area of study and/or on the use they make of the space. In this case, *A. sylvaticus* is the more widely distributed in Mediterranean environments (Castién and Gosálbez, 2001), and it is found more narrowly tied to the European wild rabbit and its shelters (Díaz, 1992). Therefore, a greater prevalence of RHDV in *A. sylvaticus* may be influenced by these factors.

The fact that the viruses detected in mice were identical (Ms8 and As15) or very similar (As26) to the viruses isolated in rabbits in the same vicinity and to the rest of the viruses that circulate in the Iberian Peninsula (Fig. 1) indicates that the strains of RHDV circulating in nature are capable of infecting different species of mammals. This finding is of great interest in view of the fact that if the non-pathogenic circulation of RHDV were demonstrated in wild mice, this would substantiate the theory that the origin of the RHDV may be in the transmission of this virus to the rabbit from another species (Fenner and Fantini, 1999). On the other hand, the prevalence and transmission of the virus within mice is not well known but it could be related to the fact that they use rabbit warrens as shelter structures (Delibes-Mateos et al., 2008), which may facilitate contact with the virus, through infected rabbits (ill or reservoir individuals).

The co-occurrence of these species of micromammals during outbreaks of the disease in the rabbits could indicate that mice may be infected passively by the impregnation of viral particles in legs and hairs, which are ingested during grooming. Another source of infection could be the ingestion of parts of the remains of rabbits that have died of the disease, especially those dead in the interior of their warrens (Cooke, 1996). Yet another possible means of infection is the faecal–oral route, as happens among rabbits (Xu and Chen, 1989). Rabbit faeces can carry the viruses both because the first viral replication occurs in the intestinal crypts of Lieberkühn (Gregg et al., 1991) and because further viruses are conducted to the intestines through the bile duct, following intrahepatic replication (Marcato et al., 1991). In this way, species that ingest the rabbit faeces, such as *A. sylvaticus and M. spretus* (Valverde, 1967), might be infected.

In this study, it has not been possible to ascertain whether the murids with virus presented an immune response, such as that induced in predatory animals or scavengers after ingesting the remains of ill or dead animals (Leighton et al., 1995; Parkes et al., 2004; Simón et al., 1994). Nor has it been possible to prove whether they can suffer from the disease or whether they have some sort of clinical manifestation due to the presence of RHDV, although at the time of their capture none showed any symptoms of the disease. Nevertheless, it would be important to determine the viability of RHDV and how long it can endure in natural murid populations. These data could inform on the intraspecific and interspecific transmission and diffusion of the virus (Merchán et al., in preparation) and on the cycles of occurrence of RHD, which could be influenced by the existence of associated micromammalian communities. It is unknown whether these micromammals have the capability to excrete and transmit the RHD virus, but were this the case, the endurance and spread of RHDV could be affected. Furthermore, as an evolutionary consequence of continuous contact with the virus, the rodents could participate in inducing a certain immune response in some individuals within sympatric rabbit populations, if the latter are infected by carrier mice.

For this reason, the presence of rodent communities in sympatry with the European wild rabbit could facilitate the permanence and generalization of these viral forms. If cross-infection between species were proved conclusively, this could suggest the existence of an ecological relationship of passive

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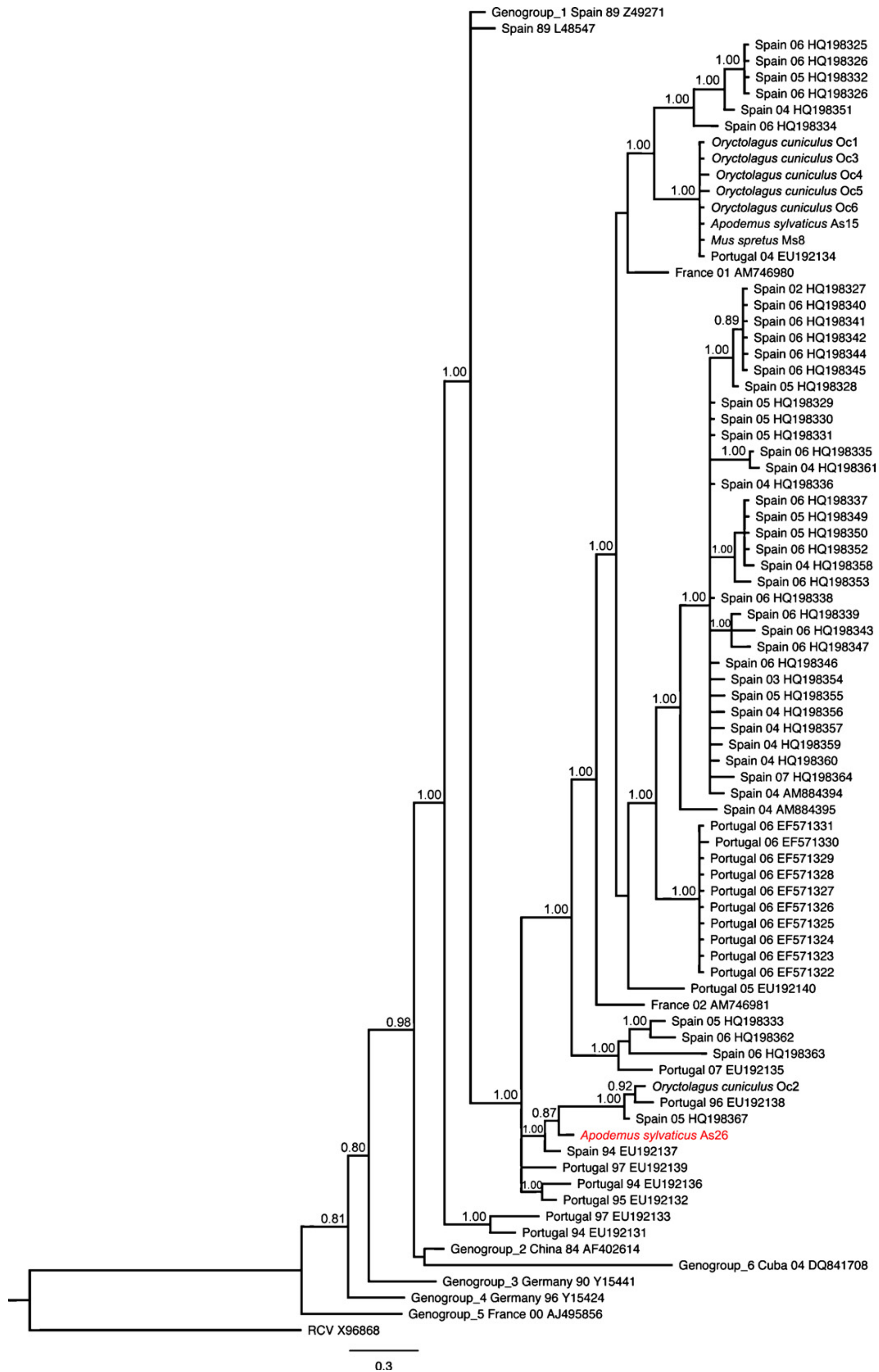

Fig. 1. Phylogenetic tree obtained by Bayesian inference for the partial VP60 gene sequences of RHDV. Numbers above branches indicate posterior probabilities above 0.80 for BI. Year and region of isolation are indicated for all samples obtained from GenBank. The sequences were grouped into genogroup 1–6 adapted to the classification used by Le Gall-Recoulé et al. (2003). RHDV isolated from non-rabbit hosts are indicated and highlighted in red. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

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collaborators in the increase of the rabbits' resistance to RHDV infections.

To conclude, we consider it important to preserve the communities of micromammals in sympatry with the European rabbit in light of the complex relationship that the populations of this species maintain with RHDV. This report clearly manifests the need for further studies to fully determine the potential of micromammals as environmental preservers of RHDV or as transmitters of less fatal varieties of the virus, especially considering the implications on the origin and the dynamics of the disease that this may have.

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