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Short communication

New insight into the epidemiology of rabbit hemorrhagic disease viruses in Portugal: Retrospective study reveals the circulation of genogroup 5 (G5) in Azores and discloses the circulation of G1 and G6 strains in mainland until 2008

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ABSTRACT

The genetic relationships between 10 rabbit hemorrhagic disease strains collected in Portugal between 2006 and 2013, originated in the mainland and Azorean islands, were investigated based on the *vp60* gene variability. A genetic diversity ranging from 2% to 13% was determined among the 10-*vp60* complete sequences revealing a significant level of genetic heterogeneity between same strains. Phylogenetic Bayesian analysis showed that the Portuguese RHDV strains fell within different genogroups, namely G1, G5 and G6. Interestingly, all strains obtained from Azores, where RHDV was first detected in 1988, belong to G5 genogroup. G5 strains, that were not identified in the continent so far, seem to be the dominant group in these Atlantic islands.

G1-related strains belonging to the Iberian group 3 ($n = 3$) and G6 (RHDVa) strains ($n = 2$) were identified among the samples originated in mainland which were collected between 2006 and 2008. Although the presence of G1 and G6 in Portugal had been shown before, our data refines the time of circulation of these strains until at least 2008.

In summary, this study revises the epidemiological information of RHDV in Portugal since it reports for the first time the presence of G5 strains in Azores and demonstrates the circulation of G1 and G6 strains in mainland Portugal until the late 2000s.

1. Introduction

Rabbit Hemorrhagic Disease (RHD) is a highly contagious and lethal infection of wild and domestic members of the species *Oryctolagus cuniculus*, caused by a single stranded positive-sense RNA, non-enveloped icosahedral virus that belongs to the genus *Lagovirus* of the family *Caliciviridae* (Parra and Prieto, 1990).

The rabbit hemorrhagic disease virus (RHDV) was first identified in 1984 in China, in rabbits imported from Germany (Lui et al., 1984) although there are molecular evidences of the occurrence of asymptomatic disease in Europe before the outbreak in China (Chasey et al., 1997; Moss et al., 2002). The first outbreak in Europe was reported in Italy in 1986 (Cancellotti and Renzi, 1991).

In Portugal the disease was first described in the summer of 1987 in Madeira Island (Neves da Costa, M. and Dória, C., *personal communication*). In the following years the disease was reported in the Azorean islands of Faial (1988), (Carvalho et al., 1994), São Jorge, (January, 1989) (Carvalho et al., 1993) and Santa Maria Island (1990) (Carvalho and Almeida, 1991). In 1990, RHD wiped out rabbit populations in most of Azores Archipelago islands (Martins, 1993). In Portugal mainland the first cases date back to 1989.

The paths of virus spreading in Europe and in the rest of the world are not precisely known (Morisse et al., 1991; Pawlikowska et al., 2010). Dissemination of the disease can be potentiated by the trade of infected live rabbits or contaminated products (Morisse et al., 1991), mechanical vectors (Barratt et al., 1998; Crosby and McLennan, 1996), fomites, rodents, hares, predators, scavengers, natural movement of wild rabbit populations or by population management actions (Guerrero-Casado et al., 2013). The importation of contaminated rabbit meat from China (Neves da Costa, M. and Dória, C., *personal communication*) was

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considered in 1987 a likely potential route of entry of virus in Madeira Island.

Based on the phylogenetic relationship between RHDV strains with different geographic origins and dates of collection, inferred by the vp60 gene variability, distinct classifications were obtained, varying in number of lineages and tree topologies (Hukowska-Szematowicz et al., 2012; Kerr et al., 2009; Le Gall-Recule et al., 2013; Pawlikowska et al., 2010).

In a study involving 104 isolates collected between 1993 and 2000 in France, a chronological pattern of genogroup emergence was observed. A relationship between genotype and geographic origin was not found since strains did not group depending on geographic provenance. Isolates collected in France between 1987 and 1990 clustered in G1 and G2 genogroups. These strains were later replaced by G3 strains from which G4 emerged (Le Gall-Recule et al., 2003). Some strains that were initially included in G3 genogroup (Le Gall et al., 1998) were re-classified as G4 (96-Fra) and G5 (96-Wri) genogroups (Le Gall-Recule et al., 2003). G5 was considered a new independent group (Le Gall-Recule et al., 2003).

The first G5 strains were detected in 1994 in France, where they co-existed with G4 isolates until 1999. Between 2000 and 2001, G5 become the most prevalent genogroup (Le Gall-Recule et al., 2003). G6 (RHDVa) was identified in China in 1985 (AY269825) and first reported in Europe (Italy) in 1997 (Capucci et al., 1998). Independent epidemics caused by G6 strains were described all over the world, in the United States (McIntosh et al., 2007), Cuba, Korea, China, Japan, Reunion Island, and Europe (Capucci et al., 1998; Farnos et al., 2007; Le Gall-Recule et al., 2003; Schirrmeier et al., 1999; Tian et al., 2007; Yang et al., 2008).

The genogroups described in continental Portugal were far less variable. Based on partial sequences of the *vp60* gene, 40 strains (1994–2007) were found more closely related to genogroup 1 than with any others, splitting into three distinct Iberian groups (Muller et al., 2009). Two strains, obtained in 1995 (JX886002) and 2006 (JX886001) from wild European rabbits from Portugal, were recently included into G1, which is known to have persisted only in Iberian Peninsula (Abrantes et al., 2012). It was suggested that the Pyrenees might have offered an effective physical barrier between wild rabbit populations contributing to the G1 geographic segregation and maintenance in the most Western Europe (Muller et al., 2009). G6 genogroup was reported in the North of Portugal in January 2007 (Muller et al., 2009) but a partial vp60 sequence from this case was only made available in 2012. No other genogroups were reported in the country.

Related but genetically distinct from RHDV, rabbit caliciviruses (RCV) have also been described worldwide (Italy (Capucci et al., 1996), France (Le Gall-Recule et al., 2011), England (Forrester et al., 2009), Australia (Strive et al., 2009) and America (Bergin et al., 2009)). The majority of RCV strains are avirulent or of low virulence. Phylogenetic studies showed that RCVs do not group within any of the known RHDV genotypes (Kerr et al., 2009). Also, in 2010, a new variant of RHDV (RHDV2) was described for the first time in France (Le Gall-Recule et al., 2011). In the following years RHDV2 spread throughout several European neighbouring countries, namely Italy (Dalton et al., 2012), Spain (Le Gall-Recule et al., 2013), Portugal (Abrantes et al., 2013), England and Wales (Westcott et al., 2014), and Scotland (Baily et al., 2014). This variant is genetically distinct from the classical RHDV strains, clustering independently in a different phylogenetic group (Le Gall-Recule et al., 2011).

The aims of the present study were to investigate the genetic variability of 10 RHDV strains collected in mainland (in 2006 and 2008) and in the Azorean islands (in 2006, 2009 and between 2011 and 2013), and infer about the epidemiological dynamics of RHDV in Portugal. In this work, we followed the six-genogroup classification (G1 to G6) suggested by Le Gall-Recule et al. (2003, 2013).

2. Materials and methods

2.1. Amplification and sequencing of vp60 gene

Ten liver samples from wild ($n = 5$) and domestic rabbits ($n = 5$), previously identified RHDV RNA-positive in routine diagnosis by means of the method published by Tham and collaborators (Tham et al., 1999), were selected for further analysis. Samples originated from Azores and mainland Portugal (Table 1). cDNA was synthesized using the RT step of QIAGEN Long Range 2 Step RT-PCR Kit (Qiagen, Hilden, Germany). Reverse transcription was performed at 42 °C for 90 min. Information on the primers used in this study is shown in Table 2.

Amplification of the full *vp60* gene was performed with 5 μl of cDNA and 25 pmol of each primer, using the High Fidelity PCR Master Mix (Roche Diagnostics GmbH, Mannheim, Germany), according to the manufacturer's protocol and included an initial denaturation at 95 °C for 5 min, followed by 50 cycles of denaturation at 95 °C for 15 s, annealing at 50 °C for 30 s and extension at 72 °C for 2 min. Fragments of about 1700 bp were excised from agarose gels after electrophoresis and purified with the NZYGelpure (Nzytech genes & enzymes, Lisbon, Portugal).

DNA sequencing was performed using a BigDye Terminator cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. The *vp60* nucleotide sequences of the 10 RHDV strains were determined on an automated 3130 Genetic Analyzer system (Applied Biosystems, Foster City, CA, USA), and submitted to GenBank database (Table 1).

2.2. Phylogenetic analysis based on the vp60 gene

Multiple alignments were generated by CLUSTALW (Thompson et al., 1994) and the result was converted to the NEXUS format using Mesquite software (Maddison and Maddison, 2009). A total of 126 *vp60* sequences from all genotypes were retrieved from GenBank to allow a more clear view of the position that strains from Portugal occupy in the phylogenetic tree.

The phylogenetic trees based on the complete and partial nucleotide sequences of the *vp60* gene were obtained with a Bayesian inference of phylogeny throughout the MrBayes v3.1.2 software that uses the Markov chain Monte Carlo (or MCMC) simulation technique to approximate the posterior probabilities of trees (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003). MrBayes analysis was performed using the GTR model (nst = 6) with gamma-shaped rate variation with a proportion of invariable sites (rates = invgamma). The analysis was run for 10^6 generations (ngen = 10^6) with four chains of temperature (nchains = 4) and each chain was sampled every 10th generations (samplefreq = 10). As outgroup an European brown hare syndrome virus (EBHSV) (Z69620) was included. EBHSV belongs to genus *Lagovirus* causing in hares a disease similar to RHDV in rabbits and is genetically distinct although related to RHDV (Le Gall et al., 1996).

2.3. Screening for polymorphism and nucleotide divergence between viral populations defined accordingly to genogroups

Genetic similarities were determined with GeneDoc software (version 2.7). Identification of polymorphic sites and DNA polymorphisms were carried out with resource to DNASPv5 (Librado and Rozas, 2009). Also using this software, the extent of nucleotide divergence within RHDV genogroups was measured.