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Tracking the evolution of the G1/RHDVb recombinant strains introduced from the Iberian Peninsula to the Azores islands, Portugal

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ABSTRACT

Previous genetic characterization of rabbit haemorrhagic disease virus (RHDV) from Azores, Portugal, revealed the presence of genogroup 3–5 (G3–G5) like strains. These strains differed from the genogroup 1 (G1) strains circulating in mainland Portugal, suggesting an independent evolution of RHDV in Azores. More recently, the new variant RHDV (RHDVb) was detected in Azores. In mainland Portugal, current circulating strains resulted from recombination events between RHDVb and non-pathogenic or pathogenic G1 strains. To characterize the RHDVb strains from Azores, a ~2.5 kb fragment of the RHDV genome (nucleotide positions 4873–7323), including the complete sequence of the capsid gene VP60 (nucleotide positions 5305–7044), was amplified and sequenced. Samples were obtained from rabbits found dead in the field between December 2014 and March 2015 in the Azorean islands Flores, Graciosa, São Jorge, Terceira, Faial, Pico, São Miguel and Santa Maria. For VP60, the highest homology was found with Iberian RHDVb strains, while the upstream fragment revealed high similarity (~95%) with Iberian G1 strains. Phylogenetic reconstruction based either on VP60 or VP10 grouped the Azorean strains with Iberian RHDVb strains. For the fragment upstream of VP60, the Azorean strains grouped with G1. Our results show that the RHDVb strains circulating in Azores are G1/RHDVb recombinants and we hypothesize that such strains had their origin in Iberian strains. The geographic isolation of Azores suggests that arrival of RHDVb was man-mediated. A network analysis further allowed us to trace virus dispersion in Azores: from an initial outbreak in Graciosa, RHDVb spread to São Jorge and Faial, to Terceira, Flores and Santa Maria, and finally to Pico; dispersion to São Miguel occurred later from Terceira. As the consequences of the presence of G1/RHDVb strains in Azores are unpredictable, we suggest a continued monitoring and characterization of RHD outbreaks.

1. Introduction

In the Iberian Peninsula, two refuges originated two European rabbit subspecies: *Oryctolagus cuniculus algirus* and *Oryctolagus cuniculus cuniculus* (Ferrand and Branco, 2007). The subspecies *O. c. cuniculus* is present throughout Europe, Australia and South America and originated all rabbit domestic breeds (Ferrand and Branco, 2007). The subspecies *O. c. algirus* is restricted to the south-western part of the Iberian Peninsula, and the islands of Azores, Madeira and Canary (Ferrand and Branco, 2007). In the last 60 years, the Iberian wild rabbit populations suffered a great decline, mainly due to two viral diseases, myxomatosis and rabbit haemorrhagic disease (RHD) (Delibes-Mateos et al., 2014b). RHD is caused by a calicivirus, the rabbit haemorrhagic disease virus (RHDV), that was first reported in China in 1984 and rapidly spread worldwide, reaching the Iberian Peninsula in 1988 (reviewed in Abrantes et al., 2012b).

The study of the genetic diversity of RHDV allowed the distinction of six genogroups, G1–G6 (Le Gall-Recule et al., 2003). Until 2011, only G1 strains were recovered from Iberian wild rabbit populations (Abrantes et al., 2012a; Alda et al., 2010; Muller et al.,

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2009); G6 (RHDVa) has also been detected, sporadically, in domestic rabbits (Abrantes et al., 2014; Duarte et al., 2014). This epidemiological pattern contrasts with that from other European countries where G1 was successively replaced by strains from other genogroups (Le Gall-Recule et al., 2003). Recently, a new variant of RHDV, named RHDV2 or RHDVb, was described in France (Le Gall-Recule et al., 2011). This new variant has unique features and dispersed quickly throughout Europe and now circulates in Spain, Portugal, Italy and United Kingdom (Abrantes et al., 2013; Baily et al., 2014; Dalton et al., 2012; Le Gall-Recule et al., 2013; Simpson et al., 2014). In the Iberian Peninsula, RHDVb reduced rabbit numbers in ~80% (Delibes-Mateos et al., 2014a). Characterization of full genomic sequences of Iberian RHDVb strains showed that these strains differ ~15% in the capsid and ~22% outside of the capsid from the other pathogenic and non-pathogenic strains (Dalton et al., 2015). Lopes et al. (2015b) further showed that Iberian RHDVb strains resulted from multiple recombination events involving non-pathogenic strains and pathogenic G1 strains. Indeed, while for the gene encoding the major capsid protein, VP60, and for the gene of the minor structural protein, VP10, all RHDVb viruses clustered together and apart from all the other strains, for the region encoding the non-structural proteins these strains appeared in three different clusters. Further analysis supported the existence of a single recombination breakpoint located in the 5′ region of VP60 compatible with the recombination pattern described.

The Portuguese Azores archipelago is located in the middle of the North Atlantic Ocean and is composed by nine islands (Fig. 1). Graciosa, São Jorge, Terceira, Faial and Pico constitute the central group; Flores, along with Corvo, compose the occidental group; São Miguel and Santa Maria belong to the oriental group. The European rabbit was introduced in Azores more than 500 years ago by the Portuguese settlers. Although the presence of RHD has been detected in the late 1980s, only in 2014 the circulating strains were characterized (Duarte et al., 2014; Esteves et al., 2014). These strains were related to G3–G5 strains, but had unique features and differed at least 8% from other RHDV strains and ~15% from RHDVb, suggesting an independent evolution of RHDV in the Azorean islands after an initial introduction more than 17 years ago (Esteves et al., 2014). The presence of RHDVb was recently reported in the Azorean islands Graciosa, Terceira, São Jorge and Flores (Duarte et al., 2015). Since this report was based on a short fragment of the capsid gene (347 nucleotides, nt) and recombination clearly plays a role in shaping RHDVb diversity (Lopes et al., 2015b), we sequenced and analyzed a 2451 nt fragment, including the complete capsid gene, of RHDV strains recovered from rabbits found dead in Flores, Graciosa, Terceira, São Jorge, Faial, Pico, São Miguel and Santa Maria between December 2014 and March 2015.

2. Material and methods

2.1. Amplification and sequencing

Liver samples of 80 rabbits found dead in the field in the islands Flores ($n = 12$), Graciosa ($n = 30$), São Jorge ($n = 7$), Terceira ($n = 4$), Faial ($n = 1$), Pico ($n = 2$), São Miguel ($n = 14$) and Santa Maria ($n = 10$) were collected between December 2014 and March 2015 (Table 1), by the Direção Regional dos Recursos Florestais, Azores, and sent to the laboratory of CIBIO, InBIO-UP. No live animals were shot, trapped or handled to obtain tissues, such that no Animal Ethics permit was required. In the majority of the islands, dead rabbits were found in several places and carcasses were collected at the beginning of the outbreak (Table 1). At necropsy, the animals presented gross histopathological alterations compatible with RHD. A portion of liver sample (~30 mg) was homogenized in a rotor–stator homogenizer (Mixer Mill MM400, Retsch) at 30 Hz for 7 min. Extraction of viral RNA was performed using the

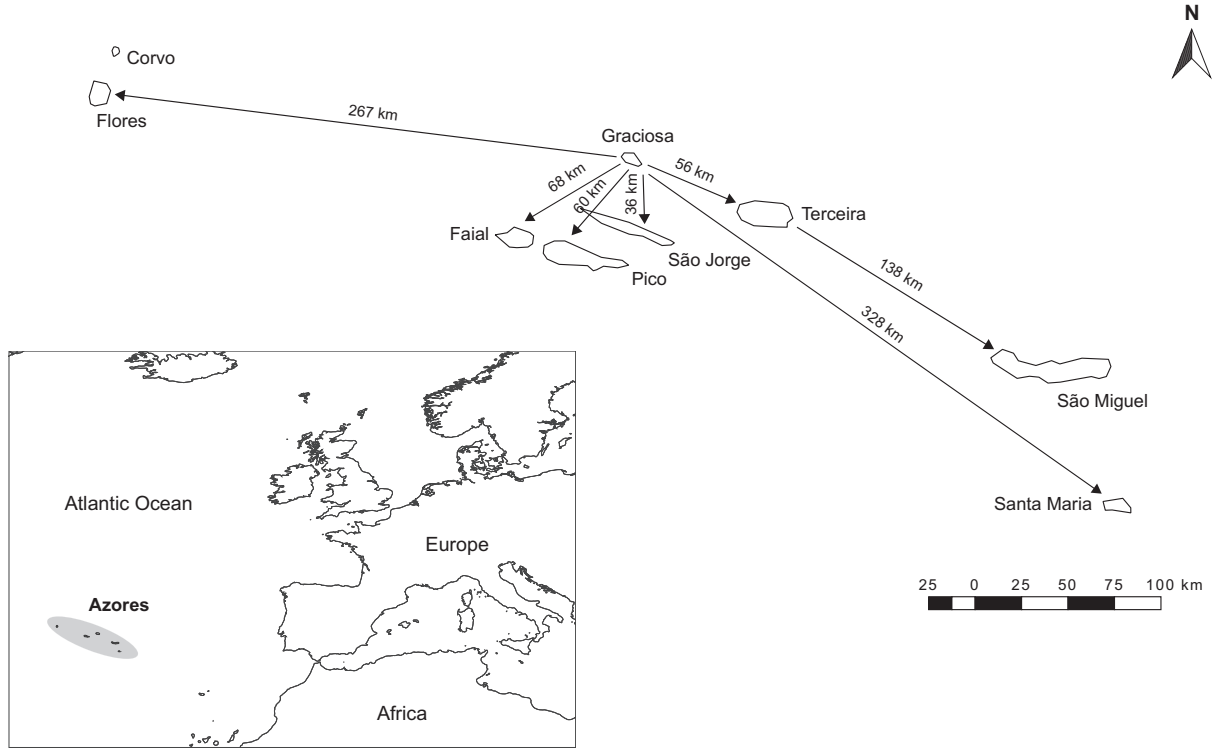

Fig. 1. Map of the Portuguese Azores archipelago. Location of the islands is shown on the insert on the left (shaded light gray). Distances between Graciosa, where the first outbreak was detected, and Flores, Terceira, São Jorge, Pico, Faial and Santa Maria are indicated as well as between Terceira and São Miguel. RHDVb presence was detected in all islands except Corvo, where no rabbits exist.