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Monitoring of the novel rabbit haemorrhagic disease virus type 2 (GI.2) epidemic in European wild rabbits (*Oryctolagus cuniculus*) in southern Spain, 2013–2017

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ABSTRACT

Rabbit hemorrhagic disease (RHD) is a highly infectious disease in European rabbits (*Oryctolagus cuniculus*), caused by a virus belonging to the genus *Lagovirus* (RHDV; family *Caliciviridae*). In 2010, a new genotype of RHDV (RHDV2 or RHDVb, currently designated GI.2) emerged in France, affecting both domestic rabbits, even those vaccinated for the classical RHDV genotypes (currently designated GI.1) and wild rabbits. GI.2 was subsequently identified in other European countries. The aim of the present study was to monitor the GI.2 epidemic in wild rabbits in Andalusia (southern Spain) during the period 2013–2017.

At the beginning of summer 2013, high mortalities were detected in wild rabbit populations in southern Spain. A total of 96 affected hunting or protected areas were surveyed. The first outbreak was observed on June 2013. The number of outbreaks sharply increased in 2013 and 2014, with a decreasing trend being observed during the following years. The spatial distribution of GI.2 was not homogeneous, since most of the detected outbreaks were concentrated in the western part of Andalusia. The outbreaks peaked in winter and spring and have been detected in the last five consecutive years, which suggests endemic circulation of GI.2 in wild rabbit populations in Spain.

A total of 190 dead rabbits from 87 of the 96 areas surveyed were collected during the study period. Mortality affected rabbits of different age classes, including kittens. RT-PCR confirmed the presence of GI.2 RNA in the livers of 185 of the 190 (97.4%) rabbits. Phylogenetic analysis performed on eleven samples collected in different provinces of Andalusia between 2013 and 2017, showed high nucleotide identity with GI.2 strains Spain, France and Portugal. The results constitute an important step in understanding of the emergence and spread of GI.2 in this country and will provide valuable information for the development of surveillance programs in Europe.

1. Introduction

Rabbit hemorrhagic disease (RHD) is a highly infectious, often fatal disease caused by the rabbit hemorrhagic disease virus (RHDV; genus *Lagovirus*, family *Caliciviridae*), which affects domestic and wild European rabbits (*Oryctolagus cuniculus*). The etiological agent is a non-enveloped, positive-sense, single-stranded RNA virus. Following the recently proposed classification by Le Pendu et al. (2017), RHD viruses are divided into four genotypes: genotype GI.1 (*Lagovirus europaeus*/GI.1a-GI.1d), which comprises pathogenic lagoviruses previously divided into phylogenetic groups G1-G6, the non-pathogenic RHDV-related viruses detected in Europe and Australia, which are classified into genotypes GI.3 and GI.4, and the novel RHDV genotype 2 (*Lagovirus europaeus*/GI.2, previously referred to as RHDV2 or RHDVb).

Pathogenic GI.1 was first described in China in 1984 (Liu et al., 1984) and has become endemic on many continents, including Europe.

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On the Iberian Peninsula, where the European rabbit is native and constitutes a keystone species in Mediterranean ecosystems (Delibes-Mateos et al., 2007), GI.1 was first identified in 1988 (Argüello-Villares et al., 1988). In the years that followed, RHDV spread rapidly and became endemic, although mortality was significantly lower (close to 30%) than the 55–75% reported during the first epidemic (Villafuerte et al., 1995). Part of the reason for the lower mortality could be the progressive increase in immune animals produced by the constant circulation of the virus in later years (Calvete et al., 2002). In this context, the prevalence of antibodies against the classical GI.1 strains in wild rabbit populations in southern Spain was found to be above 30% during the period 2003 and 2004 (García-Bocanegra et al., 2011).

The novel *Lagovirus europaeus*/GI.2 (henceforth GI.2) emerged in France in 2010, affecting both domestic rabbits, including those vaccinated against the classical GI.1 genotype, and wild rabbits (Le Gall-Reculé et al., 2011, 2013). In the years that followed, GI.2 was identified in other European countries, as well as on other continents including Australia, Africa, America and Oceania (reviewed in Rouco et al., 2019). Differences in pathogenicity between GI.1 and GI.2 lagoviruses were associated with age class (Dalton et al., 2012; Le Gall-Reculé et al., 2013); rabbits less than 5-8 weeks old were not naturally susceptible to GI.1 infection, whereas GI.2 caused disease and death even in kittens as young as 11 days of age (Dalton et al., 2014). Moreover, although hare species are naturally resistant to the classical GI.1 genotype, GI.2 cases have been detected in different hare species, including European brown hares (*Lepus europaeus*) (Bell et al., 2019; Le Gall-Reculé et al., 2017; Velarde et al., 2017), Cape hares (*Lepus capensis subsp. mediterraneus*) (Puggioni et al., 2013), Italian hares (*Lepus corsicanus*) (Camarda et al., 2014) and mountain hares (*Lepus timidus*) (Neimanis et al., 2018a).

To date, longitudinal survey studies to assess the evolution and spread of GI.2 in wild rabbit populations have only been conducted in Portugal (Rouco et al., 2016). Hence, using passive surveillance, the aim of this study was to monitor the GI.2 epidemic in wild rabbits in Andalusia (southern Spain) during the period 2013–2017.

2. Material and methods

2.1. Sampling and data collection

By the beginning of summer 2013, high mortalities were being detected in wild rabbit populations in Andalusia, southern Spain (36 °N–38°60´N, 1°75´W–7°25´W). An emergency health program was launched in this area by the Regional Ministry of the Environment of Andalusia. A total of 96 areas comprising 91 hunting areas and five protected areas in the eight provinces of Andalusia were visited by veterinarians belonging to the Epidemiological Surveillance Program for Wildlife (Fig. 1). Epidemiological information was gathered at each surveyed site by direct interview of gamekeepers and using a standardized questionnaire. Data collected included: location, date, clinical signs, date of onset in clinically affected animals, abnormal mortality in Iberian hares (*Lepus granatensis*) (the other lagomorph species present in the study area), rabbit densities before the outbreak and restocking programs.

A total of 190 rabbits found dead were sampled between June 2013 and March 2017 in 87 out of 96 areas surveyed. Individual information, including age and sex, was gathered from each animal whenever possible. Rabbits were classified according to their weight and the presence/absence of the epiphyseal notch at the head of the tibia as kittens (up to 40 days old), juveniles (from 40 days to 8 months) or adults (over 8 months) (Dalton et al., 2012; Watson and Tyndale-Biscoe, 1953). Liver samples were collected and sent to the Central Veterinary Laboratory in Algete (National Reference Laboratory for RHDV, Madrid, Spain) for the diagnosis of RHD. In the present study, the term ‘case’ was defined as a rabbit with both clinical signs and lesions compatible with RHDV infection and the presence of GI.2 RNA confirmed by real-time reverse transcription PCR (RT-PCR). The term ‘outbreak’ was defined as an area surveyed with at least one case.

2.2. Laboratory analysis

The BioSprint 96 DNA Blood Kit (Qiagen, Hilden, Germany) was used to extract RNA from 200 µl of liver homogenate (2%) in PBS, using carrier RNA by a magnetic bead robotic system during extraction to increase yield. A GI.2-specific real-time RT-PCR was then performed using the AgPath-ID™ One-Step RT-PCR kit (Applied Biosystems, Foster City, CA, USA) to detect a conserved region of the VP60 capsid protein gene of GI.2 viruses using primers (0.4 µM) sense 5’-TCCAGATGGTTTYCCTGACATG-3’ and antisense 5’-GCGGTAGGGARGGTGYTG-3’ and probe (0.15 µM) 5’-FAM-CGCTGAAGGGTACAAATG-MGB-3’ (Rocha, manuscript in preparation). The thermal profile was 48 °C for 25 min, followed by 10 min at 95 °C and 40 cycles of 2 s at 97 °C, 45 s at 55 °C. Samples negative for GI.2 RNA were further analyzed by RT-PCR assay to detect the presence of GI.1 RNA, following the protocol described by Ros Bascuñana et al. (1997).

Phylogenetic analysis was performed on partial VP60 gene sequences (from nucleotide (nt) 6227 to nt 6778. Nucleotide position refers to coordinates in RHDVAst 89 (GenBank Accession Number: Z49271)), amplified using RT-PCRs with the following pairs of primers: sense (RHNaV-F) and antisense (RHNaV-R) (Dalton et al., 2015) and sense (REF) and antisense (REB) (Ros Bascuñana et al., 1997). The amplification products were purified with the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany). Sequencing reactions were carried out using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and analyzed with a 3130XL Genetic Analyzer (Applied Biosystems). Clustal was used for nucleotide sequence alignment, using representative VP60 gene sequences of GI.1 and GI.2 from China, the Czech Republic, Germany, France, Italy, Ireland, Malta, Portugal, the United Kingdom, the United States of America and Spain, available in GenBank. An Australian GI.4 strain sequence was also included (GenBank Accession Number: EU871528). A sequence of the European brown hare syndrome virus (EBHSV) (GenBank Accession Number: Z69620), which is a highly related but phylogenetically distinct lagovirus, was used as an outgroup to root the tree. The phylogenetic tree was reconstructed with the maximum likelihood method, using the Kimura two-parameter evolutionary model implemented in MEGA 7 (Kumar et al., 2016). K2 + G was chosen as the best-fit nucleotide substitution model with the lowest BIC (Bayesian information criterion) using jModelTest 2.1.10 (Kimura, 1980; Darriba et al., 2012).

3. Results

GI.2 outbreaks were confirmed in 86 of the 96 areas surveyed between 2013 and 2017. Two rabbits from one surveyed area showed negative results for both GI.2 and GI.1 by RT-PCR. In addition, although samples from dead rabbits could not be collected in the nine remaining areas, mortality was observed by gamekeepers. The first outbreak was reported on 27 June 2013 on a hunting estate in the province of Jaen (Fig. 1). Sixteen new outbreaks were detected between November and December of the same year. There was a sharp increase in the number of outbreaks in 2014 (50; 58.1% of the total outbreaks confirmed) followed by a decreasing trend in subsequent years, (11 (12.8%) of total outbreaks confirmed in 2015, 7 (8.1%) in 2016, and one (1.2%) in 2017) (Fig. 2).

Whereas GI.2 outbreaks were detected throughout the year, 45 (52.3%) of the 86 GI.2-positive areas surveyed in Andalusia reported outbreaks during the winter, with lower frequencies being noticed in spring (14; 16.3%), autumn (24; 27.9%) and summer (4; 4.7%) (Fig. 2). GI.2 cases were found to be constant throughout the year in seven (8.3%) of the areas surveyed. GI.2 outbreaks were confirmed each year during the study period; furthermore, in 14 of the surveyed areas, GI.2

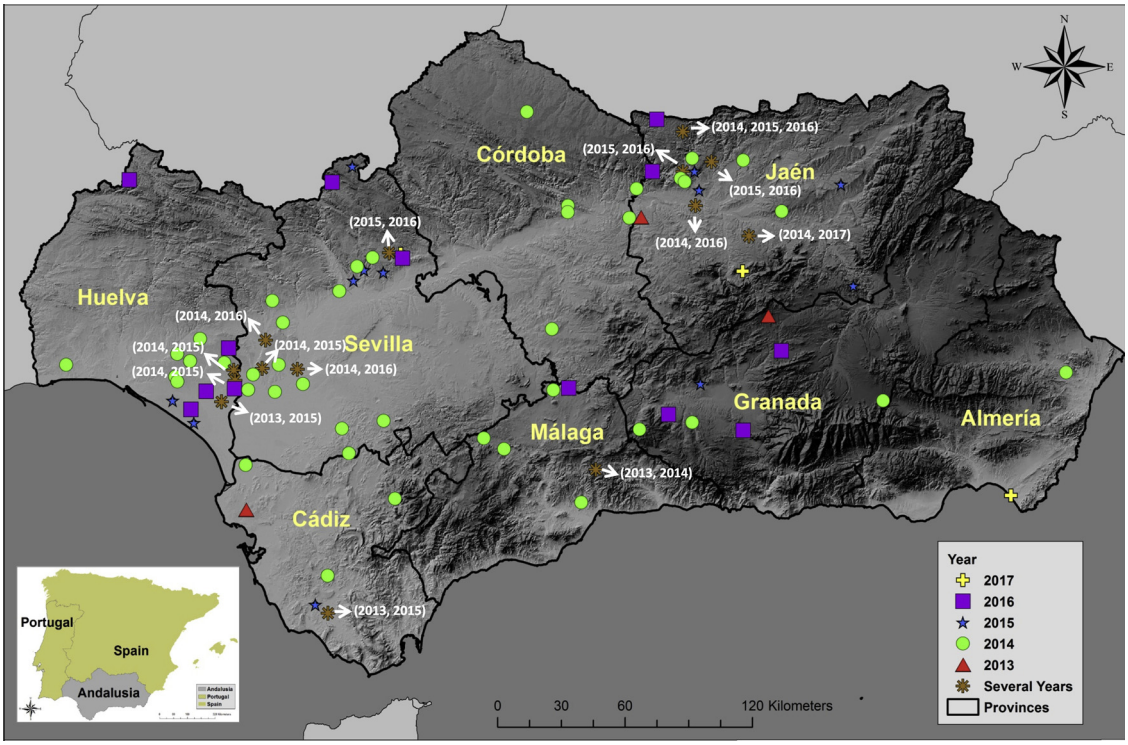

Fig. 1. Spatial distribution of GI.2 outbreaks reported in wild rabbits in Andalusia (southern Spain) between 2013 and 2017.

cases were detected in different years consecutively (Fig. 1). At least one outbreak was confirmed in all eight provinces of Andalusia. Spatial distribution was not homogeneous: Sevilla (21; 24.4%), Huelva (21; 24.4%) and Jaen (21; 24.4%) were the provinces with the highest number of outbreaks, followed by Granada (7; 8.1%), Cordoba (7; 8.1%), Malaga (5; 5.8%), Cadiz (3; 3.5%), and Almeria (1; 1.2%) (Fig. 1).

Restocking programs were conducted between six months and two years before the first GI.2 was detected in the restocked hunting area. These programs were performed in 26 of the 86 GI.2-confirmed areas located in the provinces of Cadiz, Cordoba, Huelva, Jaen, Malaga and Seville. All restocked rabbits were captured in other hunting areas in Andalusia. Vaccination was applied to restocked rabbits using single-doses of commercial inactivated vaccine against the classical GI.1 genotype, but not against GI.2. The clinical signs observed by game-keepers in kittens, juvenile and adult rabbits in the areas surveyed were sudden death (33.3%), opisthotonos (13.8%), convulsion (9.7%), ataxia (4.9%), paralysis (1.3%) and trembling (0.8%). Abnormal mortality was not observed in Iberian hare populations during the study.

GI.2 RNA was detected in 185 out of 190 (97.4%) rabbits analyzed, 26.5% of which were kittens, 39.3% juveniles, and the remaining 34.2% adults. The five GI.2 RNA-negative rabbits were also negative for GI.1 RNA. Phylogenetic analysis was performed on VP60 sequences of eleven samples collected in different provinces of Andalusia during the study period (2013: Malaga (GenBank Accession Number: MK843809) and Granada (MK843810); 2014: Sevilla (MK843807), Huelva

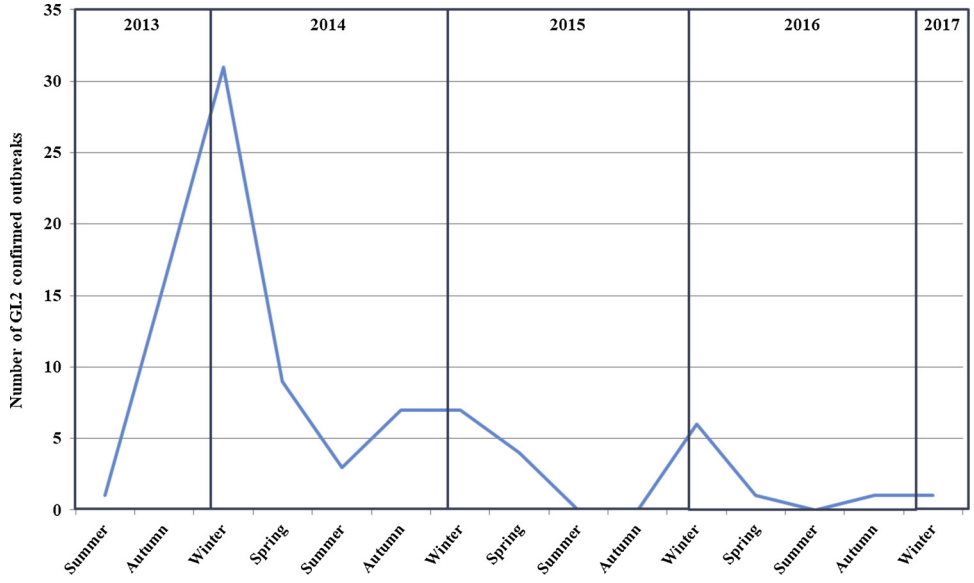

Fig. 2. Temporal evolution (by season) of GI.2 outbreaks in wild rabbits in Andalusia (southern Spain) (2013–2017).

Fig. 3. Maximum likelihood (ML) phylogenetic tree of partial VP60 sequences (n = 72 sequences; bootstrap analysis of 1000 replicates) (from nt 6227–6778 using Z49271 as reference sequence) based on the nucleotide substitution K2 + G model. The tree is drawn to scale, with branch lengths measured as the number of substitutions per site. Only bootstrap values ≥70 are shown. The branch including the sequences of GI.2 isolates identified in the present study (in bold) is enlarged.

(MK843808), Almeria (MK843811); 2015: Huelva (MK843812) and Jaen (MK843813); 2016: Cordoba (MK843814) and Granada (MK843815); 2017: Almeria (MK843817) and Jaen (MK843816)). The sequences were clustered together and included in a larger clade that comprised the GI.2 sequences from Portugal, and some isolates from Spain and France (Fig. 3). In addition, BLAST analysis showed high nucleotide identity (97–100%) with available GI.2 sequences from Spain and Portugal.

4. Discussion

The introduction of the emerging GI.2 in 2011 has led to a substantial decline in wild rabbit populations across the Iberian Peninsula (Monterroso et al., 2016). Their densities also decreased in the study area after the first GI.2 case was confirmed in 2013 (CMAOT, 2019). The presence of GI.2 outbreaks in at least 86 surveyed areas distributed across all eight provinces of Andalusia indicates the widespread dispersal of this new lagovirus in southern Spain. Nevertheless, the spatial distribution of the GI.2 outbreaks was not homogeneous, since most of them (73.2%) were concentrated in three provinces (Sevilla, Huelva and Jaen). Differences in wild rabbit population densities, variations in the surveillance efforts made to detect cases (which may have been more focused on areas with the presence of endangered species) and differences in habitat or climatic conditions are possible

factors implicated in the geographical variation observed. In this context, the distribution and prevalence of RHDV have previously been shown to be associated with environmental factors such as rainfall and temperature (Henzell et al., 2002; García-Bocanegra et al., 2011; Liu et al., 2014). Further study of the spatial distribution of GI.2 in the study area is recommended.

The temporal evolution, as well as the detection of cases in the same areas surveyed in different years, indicate the endemic circulation of GI.2 in wild rabbit populations in southern Spain between 2013 and 2017. This hypothesis is supported by the absence of restocked animals from outside Andalusia during the study period, which decreased the risk of introduction of GI.2 strains from different regions. Following confirmation of the first GI.2 case in southern Spain (Andalusia) in summer 2013, the number of outbreaks increased sharply during 2014, with a decreasing trend in the following years. This uneven temporal distribution could be explained, as was observed for GI.1 (García-Bocanegra et al., 2011) in the study area, and more recently for GI.2 in Portugal (Rouco et al., 2016), by increased population immunity due to natural immunization against the GI.2 lagovirus as a result of contact with wild strains persistently circulating in the field. Further serosurvey studies to assess the immune status of wild rabbit populations in Andalusia would provide valuable information on this point. In addition, the possibility that gamekeepers have reported fewer outbreaks to the Regional Department of Environment in the last few years cannot be ruled out either, in which case, the number of outbreaks reported in the study period may be underestimated. GI.2 outbreaks were detected in consecutive years of the 2013–2017 study period. Although outbreaks were found throughout the year, peak incidence was observed during the coldest months (between November and April), which is consistent with previous observations of GI.1 and GI.2 epidemics elsewhere (Mutze et al., 2002; Rouco et al., 2016; Villafuerte et al., 1995).

Our results show mortality in adults but also in both kittens and juvenile animals, which is consistent with what has previously been reported in domestic and wild rabbits (Dalton et al., 2012, 2014; Neimanis et al., 2018b; Rouco et al., 2016). Clinical signs observed in the present study were compatible with acute and peracute forms associated with GI.1 infections (reviewed in Abrantes et al., 2012) and, as expected, with those previously described in GI.2 infected rabbits (Abade dos Santos et al., 2017; Dalton et al., 2012; Neimanis et al., 2018b). Abnormally high mortality was not found in the Iberian hare populations in Andalusia during the study period. However, because GI.2 cases have been detected previously in European brown hares in Spain (Velarde et al., 2017), monitoring programs should also be implemented to assess the susceptibility of the Iberian hare to GI.2 infection.

Sequence analysis of isolates showed high homology (up to 97–100%) with other GI.2 strains previously isolated in Spain and Portugal. Before the GI.2 lagovirus emerged in Spain, only classical GI.1 strains were known to circulate in domestic and wild rabbits in this country (Müller et al., 2009). However, molecular studies conducted in European countries, including Spain, France, Portugal and Sweden, as well as Australia, have demonstrated that the new GI.2 genotype has replaced the GI.1 strains previously circulating in those countries (Calvete et al., 2014; Dalton et al., 2014; Le Gall-Reculé et al., 2013; Lopes et al., 2014; Mahar et al., 2018; Neimanis et al., 2018c). Our results are in accordance with this hypothesis, and all outbreaks reported between 2013 and 2017 were caused by GI.2, although GI.1 circulation in southern Spain cannot be ruled out. Although most of the restocked rabbits were immunized using commercial vaccines against GI.1, which has been shown to be only partially protective against GI.2 at best (Le Gall-Reculé et al., 2013; Dalton et al., 2014), the number of vaccinated rabbits was too limited to achieve proper population-level immunity. Additional molecular and serological studies are required to elucidate whether GI.1 is still circulating in wild rabbit populations in Spain.

Our study has several limitations that should be taken into account. Because of the difficulties associated with finding dead wild rabbits in the field, the number of outbreaks detected in the present study was probably underestimated. Secondly, although the authors made the same sampling effort during the study period, a bias in spatial distribution associated with fewer notifications of cases by gamekeepers in the last two years cannot be ruled out. Finally, we hypothesized that the temporal distribution could also be influenced by increased natural immunity against the GI.2 lagovirus in wild rabbit populations, although additional active serosurveillance is warranted to support this hypothesis.

In conclusion, our results evidence the widespread distribution of the new GI.2 genotype in wild rabbit populations in southern Spain. The outbreaks consecutively confirmed in the period 2013–2017 suggest active and endemic circulation of this new lagovirus in this region. The results obtained contribute to a better understanding of GI.2 emergence and spread and will provide valuable information for the development of risk-based surveillance programs. Further studies are needed to assess the direct impact of GI.2 on wild rabbit populations, as well as its ecological implications for other sympatric species in Mediterranean ecosystems.

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