WILDLIFE

First cases of myxomatosis in Iberian hares (*Lepus granatensis*) in Portugal

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SUMMARY

Myxomatosis was detected in Iberian hares (*Lepus granatensis*) in Portugal, October 2018, following its emergence in Spain 3 months earlier. Here, we describe the epidemiological, molecular and anatomo-histopathological data of the first two cases. Myxoma virus DNA was detected in the eyelids, nose and perineal region in both hares. It was also detected in the lungs of hare 1 and in the spleen and liver of hare 2. The genomic insertion identified in strains from Spain was confirmed in both strains suggesting a common origin for the Iberian viruses. Gross lesions in hare 1 included palpebral oedema and conjunctival mucopurulent discharge, common in both forms of the disease in rabbits. Hare 2 presented eyelid thickening with small diffuse nodules. Histopathology of the eyelids showed extracellular myxoid matrix in hare 1 and purulent dermatitis in hare 2. Both animals exhibited good body condition, suggesting a short course of the disease and higher virulence of the virus towards the Iberian hare.

BACKGROUND

Myxomatosis is a systemic infection of wild European and domestic rabbits (*Oryctolagus cuniculus*) caused by myxoma virus (MYXV), a large double-strand DNA *Leporipoxvirus* (family Poxviridae and subfamily Chordopoxvirinae)[1] first described in Uruguay in 1896.[2] The MYXV genome consists of 163 kbp and the virus replicates in the cytoplasm of infected cells.[1]

MYXV naturally infects some rabbit species of the genus *Sylvilagus*, native from South America and California, causing few clinical signs, usually an innocuous cutaneous fibroma, which persists for some weeks followed by its regression.[3–5] Only occasionally more generalised disease may occur.[4 6] Conversely, in the European rabbit, MYXV causes a generalised, and often lethal, disease characterised by swollen head, eyelids and ears, raised cutaneous lesions over the body, ears and legs, oedema of the external genitalia and anus, blepharo-conjunctivitis and mucopurulent ocular and nasal discharge (revised in Kerr *et al*[5]). Not all MYXV strains induce the formation of the typical myxoid tumours (myxomas) on the skin, which is the main characteristic of the nodular form of the disease. Amyxomatosis or atypical myxomatosis is characterised by minor cutaneous signs and intense respiratory distress.[4 7–9]

Initially, the virus caused mortality rates of 99.8% in the European rabbit populations[10] but, within a few years, slightly attenuated strains of MYXV became more dominant. Their lower virulence allowed for infected rabbits to survive longer, hence increasing the probability of mechanical viral transmission from skin lesions by mosquito and flea vectors.[11 12] Simultaneously, natural selection acted on the wild rabbit populations, resulting in the appearance of animals resistant to myxomatosis,[12 13] probably due to an effective cellular immune response.[14]

Since the introduction of MYVX in Europe in the early 1950s, and until recently, myxomatosis was only sporadically reported in the European hare (*Lepus europaeus*)[15] and in mountain hare (*Lepus timidus*)[4 10 16] even though it was considered a rabbits' disease.[17] However, in mid-2018 this scenario drastically changed. Events of mortality in Iberian hares (*Lepus granatensis*) were described in several provinces of south and central Spain.[18] Most of the animals were found dead in the same place, suggesting direct transmission of the pathogen among hares, or in a moribund state with clinical signs of blindness, weakness and disorientation.[18 19]

The genome of the virus identified in Iberian hares was recently analysed and sequenced revealing a new recombinant MYXV with an insertion of ~2800 bp in the left side of the genome.[18 19] This insertion may have resulted from recombination within the genome of MYXV[20] or between the genetic material from MYXV and a capri-poxvirus or cervi-poxvirus.[19 20] It was mapped within the M009 gene with respect to MYXV, harbouring four ORFs phylogenetically related to MYXV genes M060, M061, M064 and M065.[20]

From October onward, the disease was also registered in South of mainland Portugal . Here, we describe the epidemiological, molecular and anatomo-histopathological data of the first two cases of myxomatosis in Iberian hares detected in Portugal.

CASE PRESENTATION

In late October 2018, an adult female (hare 1) Iberian hare (*L granatensis*) that presented oedema of the eyelids and perineal area was hunted in a reserve located in the municipality of Évora, South of mainland Portugal. A few days later, in 3 November 2018, an adult male (hare 2) that showed nodules in the eyelids, nose and lips was found dead in a hunting area of the municipality of Beja, located South of Évora.

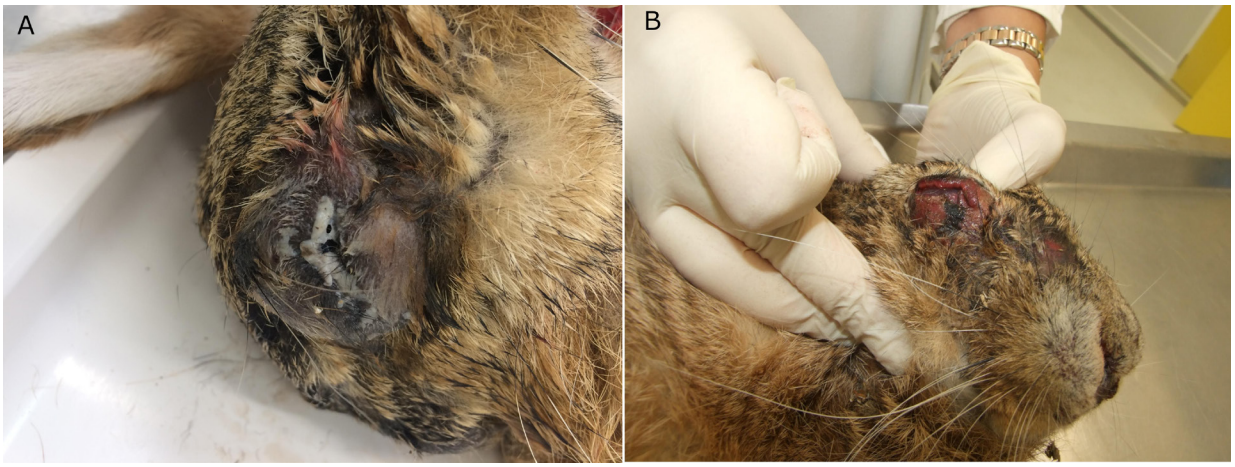

Figure 1 Macroscopic lesions found in the Iberian hares. Oedema of the eyelids and conjunctival mucopurulent discharge (A) observed in hare 1. Eyelid thickening with small diffuse nodular lesions (B) found in hare 2.

The two hares were collected and tested within the scope of an ongoing national surveillance programme on wild leporids within an action plan for the control of RHDV2 in wild rabbits (Dispatch 4757/17, 31 May, Portuguese Ministry of Agriculture).

INVESTIGATIONS

Postmortem examination and histopathology

Necropsies of both animals were carried out at the National Reference Laboratory for Animal Diseases (INIAV I.P.). Both animals were in good body condition.

Hare 1 presented conjunctival mucopurulent discharge and oedema of the eyelids (figure 1A), nose, lips and genitalia resembling the lesions observed in infected rabbits. Nodular lesions of the anal and genital mucosa were also observed . Traumatic fracture of the ribs and hemothorax were registered and attributed to the hunting shot.

In hare 2, eyelid thickening and small diffuse nodular lesions were observed in the nose, lips and eyelids (figure 1B). Pulmonary congestion was also registered.

For histopathological examination, skin samples, namely from eyelids, nose and perineal region, were fixed in 10% buffered formalin and embedded in paraffin using standard procedures. Five-micrometre-thick sections were stained with H&E and examined using light microscopy.[21]

In hare 1, eyelid lesions showed moderate epidermal hyperplasia, ballooning of epithelial cells and extensive ulceration of the epidermis with crust formation. In the dermis, proliferation of spindle and star cells surrounded by abundant extracellular matrix conferred the typical myxoid aspect. Extensive heterophilic cell infiltration in the dermal layer and intradermal pustules were also registered. Some of these features are shown in figure 2. The eyelids of hare 2 presented extensive epidermal necrosis and infiltration of the dermis where pustules with crust formation were also present.

Virological examination

For virological examination, liver, spleen, lungs and skin were homogenised with PBS and clarified at 3000 *g* for 5 min. Total DNA and RNA were extracted from 200 µl of the clarified supernatant, in a BioSprint 96 nucleic acid extractor (Qiagen, Hilden, Germany), using the MagAttract 96 Cador Pathogen kit (Qiagen), according to the manufacturer's instructions.

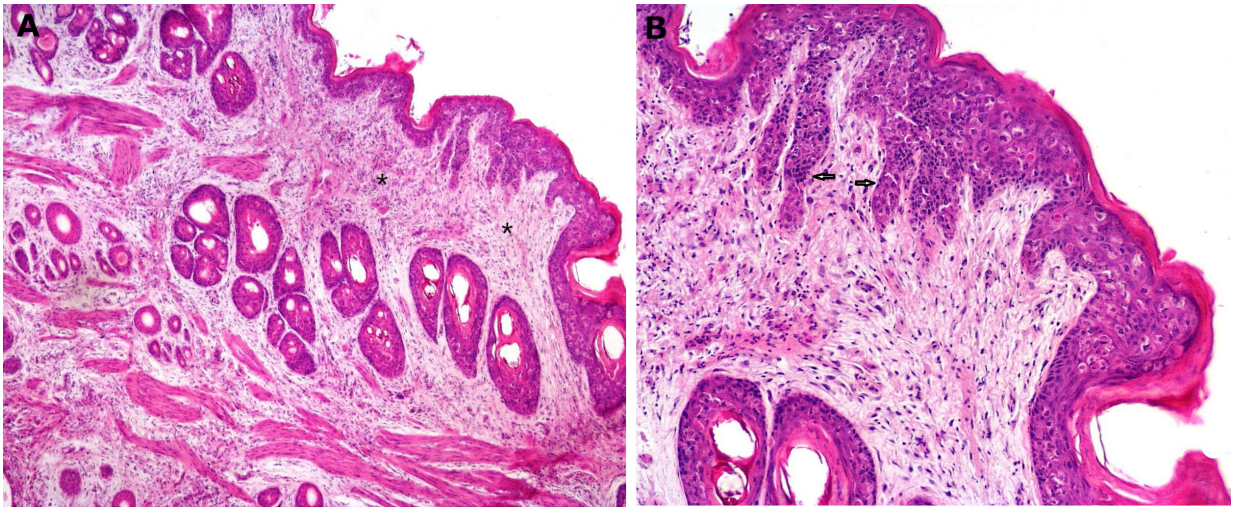

Figure 2 Histopathological features of the eyelid lesions observed in hare 1. (A) Abundant myxoid tissue indicated by black stars (x 40 magnification) and (B) hyperplasia of the epidermis pointed by arrows and the presence of myxoid tissue in the dermis (x 100 magnification). H&E staining.

The presence of MYXV DNA was investigated by using a specific qPCR targeting gene M0005R/L described by Duarte *et al*,[22] according to the assay description in the OIE manual. The FastStart TaqMan Probe Master Kit (Roche; Roche Diagnostics GmbH, Manheim, Germany) was used according to the manufacturer's instructions.

Both animals were positive for the presence of MYXV DNA in the eyelids, nose and perineal tissues in the qPCR previously described.[22] In hare 1, MYXV DNA was detected in the lungs but not in the liver and spleen, while in hare 2, viral DNA was detected in the liver and spleen but not in the lungs. Once confirmed, the results were communicated to the OIE.[23]

The animals were negative for the presence of RHDV and RHDV2 RNA by the methods described by Tham *et al*[24] and Duarte *et al*,[25] respectively.

A conventional PCR for differentiating the presence of the 2.8-kb insertion described by Dalton *et al*[20] (supplementary data table) allowed us to confirm that the MYXV strains detected in these two hares also possessed this additional genetic material in their genomes (*results not shown*).

Bacteriological and parasitological examinations

To rule out other pathogens that might have caused the death of the animals, parasitological and bacteriological examinations were also carried out by standard methods.

Oocysts of *Eimeria media* (heavy infestation) and *Eimeria magna* (light infestations) were identified by microscopic examination of the faeces of hare 1 (after flotation). Adult *Passalurus ambiguus* (light infestation) were detected by the sedimentation technique. No external parasites were detected. Only light infestations of oocysts of *E media*, strongyls eggs and *Nematodirus* species were identified in hare 2. As with hare 1, no external parasites were detected.

Bacterial examination was carried out in a pool of organs of each animal including liver, spleen and lung. *Enterococcus faecium* and *Escherichia coli* were isolated from hare 1 and hare 2 organs, respectively, using the ID 32E kit (Biomerieux). No other bacteria were found using the API 20NE kit (Biomerieux), the ID 32 STREPT (Biomerieux), the ID 32 STAPH (Biomerieux) and the API CORYNE (Biomerieux) commercial kits or standard bacteriological media (peptone water, Rappaport Vassiliadis semi solid culture media, agarose SMID2 and XLD culture media, MacConkey and blood agar culture media).

None of these results justifies the death of the animals.

DISCUSSION

Lepus granatensis is endemic to the Iberian Peninsula, and it is the only hare species found in Portugal and the most abundant in Spain.[26] Until 2018, no major threats to *L granatensis* were pointed out[27] although high hunting pressure, predation and diseases, such as tularaemia,[28] were identified as relevant factors influencing the Iberian hare population dynamics.[29] In addition, the use of rodenticides in agricultural lands and road traffic were also considered threats to this species.[30]

Here, we report the first two cases of myxomatosis in Iberian hares (*L granatensis*) in Portugal confirmed at the National Reference Laboratory in late 2018.

Molecular data gathered within a national surveillance network on wild leporids in action in the country since August 2017, show that the natural recombinant MYXV that affects hares was not circulating in Portugal prior to October 2018. Until August 2018, 80 Iberian hares were tested for myxomatosis by qPCR.[22] Of these, 79 (98.75%) were collected during the 2017–2018 hunting season, between September 2017 and February 2018, and one (1.25%) was found dead in the field. None of these hares tested positive for MYXV.[31]

The two hares investigated in this study were in good body condition, contrarily to what is common in MYXV-positive wild rabbits. The good body condition of the animals at the time of death is compatible with an acute course of disease. Moreover, the high mortality observed in the field in Iberia[18] suggests higher virulence of this new virus towards hares.

Clinically, the typical exuberant cutaneous myxomas described in rabbits[3 4 6] were not observed in the two cases reported here. García-Bocanegra *et al*[18] suggested that the form of disease in hares in Spain could be atypical amyxomatosis given the lack of myxoid tumours and the concomitant presence of pulmonary oedema and haemorrhages. However, no relation was established between the pulmonary lesions described and the presence of MYXV DNA in the lungs. Contrarily, Águeda-Pinto *et al*[19] observed lesions compatible with myxomas at the base of the left ear of one infected hare. This animal was completely emaciated suggesting a more insidious course of the disease, which may have allowed the formation of cutaneous myxomas.

Despite myxomas were not present in the two cases reported here, the typical myxoid extracellular matrix was confirmed in the skin of hare 1 (figure 2). The qPCR showed high viral loads in the skin of both hares (Cq values of 17.25 and 19.08). No viral DNA was detected in the lungs of hare 2. While the thoracic lesions found in hare 1, namely hemothorax and rib fracture, were most probably a consequence of the shot, the hemothorax and pulmonary congestion in hare 2 may have had other infectious causes than MYXV. In addition, the necrotic and infiltrative lesions observed in hare 2 of necropurulent dermatitis are not common in myxomatosis and appear to have a different origin.

None of the cases here reported are compatible with the chronic typical (nodular) myxomatosis since cutaneous myxomas were absent, and do not fit clearly in the atypical (amyxomatous) myxomatosis, since the virus was not systematically detected in the lungs. Although further investigations are necessary, the clinical presentation of the disease seems to be different in *L granatensis* and in the rabbit, suggesting therefore differences in the physiopathology of the disease in the two species.

The 2.8-kb insertion identified in the MYXV strain that is circulating in hares in Spain[19 20] was also present in the viruses from both hares. Portugal shares with Spain a long uninterrupted border of around 1200 km in length, providing many opportunities for natural movement of animals across borders, mainly in the South, where the Iberian hare is most abundant.[32]

The disease emerged in Spain in the Provinces of Cordoba (Andalusia Autonomous Community) and Cuenca (Castilla-La Mancha) and within the next weeks, the virus spread to other provinces.[33] By March of 2019, 26 spanish provinces were already affected by the disease.[33] Given the time frame of the disease emergence in both countries, it is most likely that the virus entered mainland Portugal from Spain. This may have occurred by anthropogenic factors, such as illegal movements of infected animals, fomites (namely hunters' personal equipment and/or vehicles since many hunt in both countries), or by flying insects. Arthropods such as fleas and mosquitoes are mechanic vectors of MYXV.[10 34] The high dissemination rate of the virus among the territories of Spain and Portugal suggests that flying insects, probably mosquitoes, provided the means for the rapid indirect transmission among separate hare populations.

In Portugal, from November 2018 onward, several other cases of myxomatosis in Iberian hares have been registered. To date,

six districts of mainland Portugal are affected, namely Faro, Beja, Évora, Setúbal, Santarém and Portalegre.

Although the impact of myxomatosis' emergence in the Iberian hare is yet unknown, the high mortality perceived in the field and the large number of laboratory confirmed cases may indicate that this natural recombinant MYXV is a relevant threat to the species, despite the population still being considered stable by the IUCN.[27] Investigating the physiopathology of the disease in this new species is of paramount importance to understand the clinical and epidemiological implications of this species jump event.

Learning points

- A recombinant myxoma virus (MYXV) strain was detected for the first time in Iberian hares in Portugal.
- The real-time PCR method targeting the M0005 gene of MYXV allows detection of the recombinant virus strain.
- The strains circulating in Portugal harbour the 2.8-kb insertion identified in the isolates from Spain, linking the outbreaks to a common source.
- The apparently distinct clinical signs of infection in the Iberian hare and in the rabbit suggest a different physiopathology of the disease in the two species.

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Contributors CLC carried out the experimental work regarding the virological detection and wrote the manuscript. FAAdS carried out the differentiating PCR targeting the 2.8-kb insertion. PC, PM and MM carried out the anatomo-histopathological examinations. MDD conceived the experiments, wrote and revised the manuscript. All authors discussed the results and contributed critically to the final document.

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Competing interests None declared.

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