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RAPID COMMUNICATION

Co-infection by classic MYXV and ha-MYXV in Iberian hare (*Lepus granatensis*) and European wild rabbit (*Oryctolagus cuniculus algirus*)

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ABSTRACT

Myxomatosis is an emergent disease in the Iberian hare (*Lepus granatensis*). In this species, the disease is caused by a natural recombinant virus (ha-myxoma virus [MYXV]) identified for the first time in 2018 and has since been responsible for a large number of outbreaks in Spain and Portugal. The ha-MYXV, which harbours a 2.8 Kb insert-disrupting gene *M009L*, can also infect and cause disease in wild and domestic rabbits, despite being less frequently identified in rabbits.

During the laboratory investigations of wild leporids found dead in Portugal carried out within the scope of a Nacional Surveillance Plan (Dispatch 4757/17, MAFDR), co-infection events by classic (MYXV) and naturally recombinant (ha-MYXV) strains were detected in both one Iberian hare and one European wild rabbit (*Oryctolagus cuniculus algirus*). These two cases were initially detected by a multiplex qPCR detection of MYXV and ha-MYXV and subsequently confirmed by conventional PCR and sequencing of the *M009L* gene, which contains an ha-MYXV-specific insertion.

To our knowledge, this is the first documented report of co-infection by classic MYXV and ha-MYXV strains either in Iberian hare or in European wild rabbit. It is also the first report of infection of an Iberian hare by a classic MYXV strain. These findings highlight the continuous evolution of the MYXV and the frequent host range changes that justify the nonstop monitoring of the sanitary condition of wild Leporidae populations in the Iberian Peninsula.

KEYWORDS
European wild rabbit, ha-MYXV, Iberian hare, *Lepus granatensis*, myxoma virus, myxomatosis, MYXV, *Oryctolagus cuniculus algirus*

1 | INTRODUCTION

Myxoma virus (MYXV) is a *Leporipoxvirus* from the Poxviridae family that causes an infectious and systemic disease, often fatal in the European wild rabbit, called myxomatosis. This enveloped virus has a brick-shaped morphology and a large, double-stranded DNA genome and replicates in the cytoplasm of infected cells (ICTV, 2011). After the introduction of the virus in France, in 1952, MYXV spread across Europe, resulting in mortality rates as high as 90% (Fenner & Ratcliffe, 1965). Currently, the disease persists in the Iberian Peninsula (Duarte

et al., 2018, 2021) and is considered one of the main causes behind the fragile conservation status (endangered) of the European wild rabbit (Villafuerte & Delibes-Mateos, 2019).

For almost seven decades, myxomatosis was considered to be a rabbit disease, only rarely reported in European brown hares (Collins, 1955; Wibbelt & Frolich, 2005). Myxomatosis was not reported in the Iberian hare until 2018, when a natural recombinant MYXV (ha-MYXV) likely emerged in Spain, leading to epidemic outbreaks in this species throughout the Iberian Peninsula (Carvalho et al., 2020; Dalton et al., 2019; García-Bocanegra et al., 2019). The change in host tropism (rabbit to hare) was attributed to a genomic modification comprising a novel region of ~2.8 kb (Dalton et al., 2019; Pinto et al., 2019) and possibly other mutations found throughout the genome. The additional region contains genes with homology to the MYXV *M060R*, *M061R*, *M064R* and *M065R* (Dalton et al., 2019; Pinto et al., 2019) and *M066R* genes (Abade dos Santos et al., 2020), which are located around position 60 kb in the complete genome of the Lausanne strain (KY54879). The ~2.8 kb insert disrupts the *M009L* gene (located around position 12 kb in the Lausanne strain KY54879), which becomes divided into two smaller open reading frames (ORFs) flanking the insert. Recently, the natural recombinant virus (ha-MYXV) was also reported in wild and domestic rabbits with signs of myxomatosis (Abade dos Santos, et al., 2020; Abade dos Santos, Carvalho, Pinto, et al., 2020).

In addition to the ~2.8 kbp insertion, the genome of ha-MYXV contains an additional 110 mutations when compared to MYXV. It is not yet known how many of these mutations are stable in ha-MYXV or required for the infection of the Iberian hare. One mutation that seems important due to the nature of the gene it affects is a single nucleotide polymorphism (SNP), namely, a cytosine insertion at position 147,868 bp (Lausanne strain), that disrupts ORF *M152R* (Dalton et al., 2019; Pinto et al., 2019).

Here, we report the detection of co-infection events by classic MYXV and ha-MYXV in one Iberian hare (*Lepus granatensis*) and one European wild rabbit (*Oryctolagus cuniculus algirus*). To the best of our knowledge, this is also the first report of a classic MYXV strain infecting an Iberian hare.

2 | MATERIALS AND METHODS

2.1 | Case presentation, necropsy and sample collection

This study focuses mainly on two wild leporids, an Iberian hare (31402PT21, hereafter referred to as hare-1) and a European wild rabbit (31401PT21, hereafter referred to as rabbit-1) admitted to the Wildlife Rehabilitation and Research Centre of Ria Formosa and submitted within the scope of Project +Coelho for laboratorial diagnosis at the National Reference Laboratory of Animal Health, Portugal (INIAV, I.P.). Hare-1 was a juvenile female captured in Santa Maria, Tavira (Faro, Algarve), admitted alive on 26 May 2021, with average body condition (1.075 kg) and signs of head trauma (blood contamination of the nasal region and bleeding of the right eye), dying 48 h later. Rabbit-1 was an adult female found in Luz de Tavira, Tavira (Faro, Algarve), with poor body condition (0.781 kg), oedema of eyelids, lips and external genitalia admitted alive on 20 September and euthanized at admission given its severe condition. Seven other European wild rabbits and one Iberian hare were found dead or sick in the same geographic region during the spring and summer of 2021.

All the animals were necropsied according to routine procedures in the Portuguese INIAV, I.P. Tissue samples (spleen, lung, eyelid and genitalia) were collected for virological examination. Lung, liver and spleen samples were collected for bacteriology, which was performed according to routine procedures. For parasitological examination, liver and intestinal contents were collected.

2.2 | Molecular analysis

For nucleic acid extraction, fresh samples of spleen, lung, eyelid and genitalia were homogenized at 5% (w/v) with phosphate-buffered saline using mechanical homogenisation with 0.5-mm zirconium beads (Sigma–Aldrich) using four cycles of 15 s at 3000 rpm with an interval of 10 s (Precellys Evolution) and then clarified at 3000 × g for 5 min. Total nucleic acid was extracted from 200 μl of the clarified supernatants using the MagAttract 96 cador Pathogen Kit in a BioSprint extractor (Qiagen) according to the manufacturer's protocol.

The leporids were tested for rabbit haemorrhagic disease virus 2 (RHDV2) by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) (Duarte et al., 2015) and for RHDV by conventional RT-PCR (Tham et al., 1999) using the One-step RT–PCR kit (Qiagen). MYXV was tested by a multiplex qPCR method (Abade dos Santos et al., 2021), which detects and differentiates MYXV from ha-MYXV, using the Multiplex PCR NZYTaq 2x Colourless Master Mix (NZYTech), following the conditions recommended in that study. Hares were also tested for leporid gammaherpesvirus 5 (Abade dos Santos et al., 2020) by real-time PCR (Abade dos Santos et al., 2021)

To confirm the results obtained by qPCR and to allow sequencing analyses, the conventional PCR system seq9E/seq9F described by Dalton et al. (2019) was performed using High Fidelity PCR Master Mix (Roche) according to the manufacturer's protocol. The PCR products were visualized in 2% horizontal electrophoresis agarose gels, purified with the NZYGelpure kit (Nzytech) and directly sequenced using the ABI Prism BigDye Terminator v3.1 Cycle sequencing kit on a 3130 Genetic Analyser (Applied Biosystems).

The nucleotide sequences obtained were assembled using Seqscape Software v2.7 (Applied Biosystems) and submitted to GenBank. The PCR amplifications, as well as sequencing reactions, were carried out in a Bio-Rad CFX96 Thermal Cycler (Bio-Rad Laboratories) or a QuantStudio 5 Real-Time PCR System (Applied Biosystems).

2.3 | Development of an SNP PCR + HRM

None of the sequences from classic strains currently available (six sequenced by our team and 98 available in public databases) contains

the additional C in the *M152R* gene, contrary to all characterized ha-MYXV strains (22 sequenced by our team and two publicly available), where this additional cytosine is conserved (*results not shown*). We used the specific ha-MYXV insertion and the disruption of *M152R* as specific markers for ha-MYXV and used them to differentiate MYXV and ha-MYXV.

A PCR system followed by high-resolution melting analysis was developed and optimized to easily identify an SNP comprising the presence/absence of a cytosine in gene *M152R* to distinguish between the classic and recombinant MYXV and provide an additional molecular tool for the follow-up of ha-MYXV evolution. This *M152R* HRM SNP PCR system is based on the amplification of a 62 bp (classical MYXV) or 63 bp (ha-MYXV, cytosine insertion) region by primers M152R-Fw 'AAAACAAAGTATACATAAACGCG' and M152R-Rv 'CGGTACATCGTACGCACAC'.

The commercial kit MeltDoctor HRM Master Mix (Thermo Fisher Scientific) was used by combining 10 μl of mix, 0.6 μM of each primer and 20 ng of genomic DNA in a final 20-μl reaction. The protocol followed comprised an initial denaturation at 95°C for 10 min followed by 40 cycles of denaturation at 95°C for 10 s, annealing at 52°C for 15 s and extension at 72°C for 15 s. Following PCR, a melt curve/dissociation analysis was carried out with denaturation at 95°C for 10 s and annealing at 60°C for 1 min. High-resolution melting was carried out at 95°C for 15 s with a ramp rate of 0.025°C/s and annealing at 60°C for 15 s. Reactions were performed on a QuantStudio 5 Real-Time PCR System. Eight samples of classic MYXV and eight samples of ha-MYXV, previously analysed for the presence or absence of cytosine insertion, were used to validate the method. Spiked samples combining equimolar amounts of MYXV-DNA and ha-MYXV-DNA were used to mimetize co-infections.

3 | RESULTS

3.1 | Necropsy, bacteriology and parasitology

The bacteriological (aerobic bacteria) analyses were negative for all animals, excluding the contribution of pathogenic bacteria to the death

TABLE 1 Necropsy, parasitology and bacteriology data from the rabbits and hares

Identification	Geographic origin	Necropsy data	Parasitology
Hare-1 31402PT21	Santa Maria, Tavira 26/05/2021	Juvenile female, average body condition (1.075 kg); blood contamination of the nasal region; bleeding from the right eye; subcutaneous haemorrhages in the ventral cervical region	Infection by *Eimeria* spp. and *Cestoda* oocists
Rabbit-1 31401PT21	Luz de Tavira, Tavira 20/09/2021	Adult female, poor corporal condition (0.781 kg); tumefaction of eyelids, lips and external genitalia	Mild infection by *Passalurus ambiguous* and *Anoplocephalidae*, severe infection by *Eimeria* spp. oocists
Rabbit-2 31400PT21	Fuseta, Olhão 29/05/2021	Juvenile, male, average body condition (0.397 kg); blood contamination of the nasal region; swelling of the external genitalia; congestion and pulmonary haemorrhages	Mild infection by *Anoplocephalidae*
Rabbit-3 31403PT21	Quelfes, Olhão 22/04/2021	Juvenile female, good body condition (0.848 kg); blood contamination of the nasal region; extensive subcutaneous haemorrhages resulting from trauma, congestion and pulmonary haemorrhages	Mild infection by Cestoda
Rabbit-4 31404PT21	Castro Marim, Castro Marim, 05/06/2021	Juvenile male, good body condition (0.362 kg); nodular thickening on the eyelids, nose and lips; swelling of the external genitalia	Mild infection by Cestoda
Rabbit-5 31405PT21	Moncarapacho, Olhão 08/06/2021	Juvenile female, medium body condition (0.098 kg); pulmonary congestion and liver discolouration	Negative
Hare-2 31406PT21	Monte Gordo, Vila Real de St. António 10/06/2021	Juvenile female?; Body condition(0.101 kg); Many excoriations on the skin; focus of lung congestion	Negative
Rabbit-6 31407PT21	Moncarapacho, Olhão 18/06/2021	Juvenile female, good body condition (0.117 kg); extensive subcutaneous haemorrhage in the right costal wall	Mild infection by Cestoda
Rabbit-7 31408PT21	Odemira, Odemira 20/06/2021	Juvenile male, good body condition (0.197 kg); blood contamination of the nasal region; pulmonary congestion; liver discolouration	Negative
Rabbit-8 37944PT21	Santa Maria, Tavira 04/08/2021	Male, good body condition (0.749 kg); subcutaneous bleeding in the abdominal wall; congestion and pulmonary haemorrhage; extensive bleeding in the lumbar muscles	Mild infection by Cestoda

TABLE 2 Summary of myxoma virus (MYXV) results

Specimens	Laboratorial diagnosis			Final diagnosis
	M0005 L/R qPCR	M009L qPCR	M060L qPCR	
Hare-1 (31402PT21)	+	+	+	Co-infected with ha-MYXV and classic MYXV
Rabbit-1 (31401PT21)	+	+	+	
Rabbit-2	+	+	–	Infected with classic MYXV
Rabbit-3	–	–	–	Negative to myxomatosis
Rabbit-4	+	+	–	Infected with classic MYXV
Rabbit-5	+	+	–	Infected with classic MYXV
Hare- 2	–	–	–	Negative to myxomatosis
Rabbit-6	–	–	–	Negative to myxomatosis
Rabbit-7	–	–	–	Negative to myxomatosis
Rabbit-8	–	–	–	Negative to myxomatosis
Rabbit-9	+	–	+	Infected with natural recombinant MYXV (ha-MYXV)

of the animals. A remarkable infection by *Eimeridae* oocists and low-level infections by *Passalurus ambiguus* and *Anoplocephalidae* were found in rabbit-1. In hare-1, moderate infection by *Eimeridae* oocists was detected. All the data from origin, necropsy and parasitology for all the animals are disclosed in Table 1.

All animals (hare-1, wild rabbit-1 to 9) tested negative for RHDV and RHDV2, ruling out possible co-infections by RHDV2 and MYXV previously reported (Carvalho et al., 2020), and the hares tested negative for LeHV-5 (Abade dos Santos et al., 2020; Abade Dos Santos et al., 2021).

3.2 | Molecular analysis

Hare-1 and rabbit-1 tested positive for both MYXV and ha-MYXV, in contrast to rabbits 2, 4 and 5, which were only positive for MYXV, and rabbit-9, which was only positive for ha-MYXV (Table 2). Given the novelty of the findings, we ruled out any possibility of contamination by repeating the extraction and qPCR from all samples from rabbit-1 and hare-1. The results obtained corroborated the previous findings.

Figure 1 illustrates the simultaneous amplification by the M060L system (designed to specifically detect ha-MYXV strains) and M009L system (designed to specifically detect classic MYXV strains) in samples from rabbit-1 and hare-1. In rabbit-1, ha-MYXV DNA was detected in the spleen, lungs, eyelid and genitalia, while classic MYXV DNA was detected in the eyelid and external genitalia. In hare-1, ha-MYXV and classic MYXV were detected in the eyelid and lungs.

Except for the lungs from hare-1, in both animals, the viral loads of the classic MYXV strain were lower than the viral loads of ha-MYXV. The estimated viral loads for both viruses found in the different organs are shown in Table 3.

The conventional PCR system described by Dalton et al. (2019) was used to amplify the genomic region containing the insert. Two different bands were detected by agarose gel electrophoresis, one of 3140 bp (corresponding to the insert plus flanking regions) and another of 303 bp (corresponding to the insertion within M009L; Figure 2). The

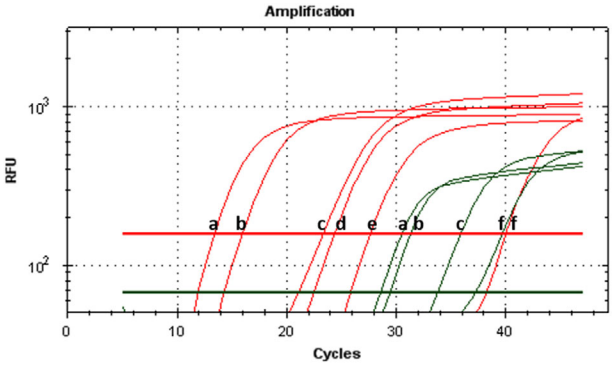

FIGURE 1 Multiplex real-time PCR amplification curves of rabbit-1 and hare-1. Red curves (TexasRed channel) correspond to amplification with the M060L system, and green curves (HEX channel) correspond to amplification with the M009L system. Rabbit-1 samples: eyelid (a); external genitalia (b); spleen (d); lungs (e). Hare-1 samples: eyelid (c); lungs (f). The M005L and 18S systems were hidden for better visualisation of the M060L and M009L curves

PCR was then repeated under a reduced extension time (15 s) to favour the amplification of the 303 bp fragment. Under these conditions, only the 303 bp-long fragment was produced (Figure 2, Lanes 4 and 5). The two fragments (3140 bp and 303 bp) from rabbit-1 and hare-1 were purified using the NZY GelPure kit (NZYTech) and sequenced using the seq9e/9f primers and other primers previously described (Abade dos Santos et al., 2020; Dalton et al., 2019), allowing the confirmation of ha-MYXV and classic MYXV simultaneous presence in both animals.

The 259 bp sequenced from hare-1 (OL979472) and rabbit-1 (OL979471) showed 100% identity with each other and with the Lausanne strain (KY548791). The ha-MYXV sequences from rabbit-1 (OL979473) and hare-1 (OL979474) comprising the 2.8 Kb insert showed 100% identity with each other, 100% identity with a previously published hare ha-MYXV sequence (MT072320) and 99.9% identity with ha-MYXV previously isolated from rabbits (MT940240 and MT940239). Both viruses (ha-MYXV and classic MYXV) were isolated

TABLE 3 Estimated viral charges (corresponding to viral DNA copies) in tissues from rabbit-1 and hare-1

		Viral DNA copies/mg tissue			
		Spleen	Lung	Eyelid	External genitalia
Rabbit-1	ha-MYXV	5.83E + 05	6.87E + 04	8.17E + 08	1.47E + 08
	Classic MYXV	N	N	8.54E + 03	4.81E + 03
Hare-1	ha-MYXV	N	1.87E + 01	1.16E + 06	NT
	Classic MYXV	N	2.79E + 01	2.73E + 02	NT

Note: NT, not tested; N, negative.

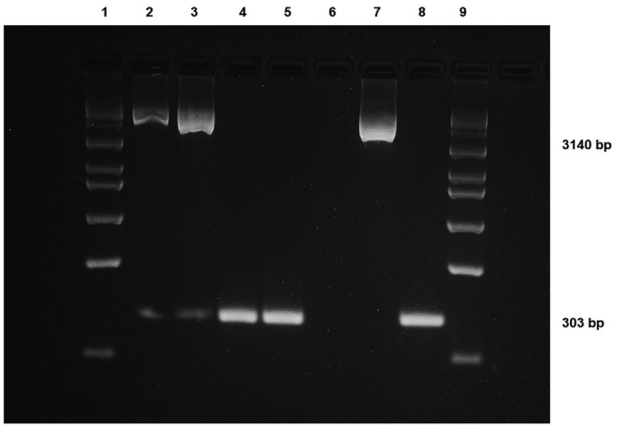

FIGURE 2 Agarose gel electrophoresis showing the amplification products obtained with the seq9E/seq9F PCR system. Lane 1–DNA markers. Lane 2–rabbit-1 (eyelid), extension of 3 min; Lane 3–hare-1 (eyelid), extension of 3 min; Lane 4–rabbit-1 (eyelid), extension of 15 s; Lane 5–hare-1 (eyelid), extension of 15 s; Lane 6–negative control; Lane 7–positive control (natural recombinant myxoma virus [ha-MYXV]); Lane 8–positive control (MYXV); Lane 9–DNA markers

in RK13 cells from rabbit-1 and hare-1 according to to previously described methods (Abade dos Santos et al., 2020), proving the virus viability.

3.3 | HRM analysis

The analyses of the single nucleotide mutation showed an average difference of 0.33°C in the melting temperature of the two different fragments obtained. While the classical viruses showed a melting temperature of 77.208 ± 0.116°C, with a minimum of 76.986°C and a maximum of 77.358°C, the ha-MYXV viruses exhibited a melting temperature of 77.539 ± 0.074°C, with a minimum of 77.431°C and a maximum of 77.707°C.

With respect to the co-infected leporids, sample 31401PT21 presented an average melting temperature of 77.423 0± 0.082°C (using eight replicates), and sample 31402PT21 presented an average melting temperature of 77.415 ± 0.052°C (using eight replicates). The melting curves obtained from the co-infected samples were located between the curves generated from the pure samples (Figure 3).

The mean melting temperature for co-infected was closer to the minimum value found in ha-MYXV samples rather than to the maximum value found in classic MYXV samples, probably due to a higher initial amount of ha-MYXV in the tissue samples. Spiked samples with a 50:50 ratio of both viruses (MYXV and ha-MYXV) generated intermediate curves corresponding to an average melting temperature of 77.398 ± 0.044°C. This method proved to be very effective in the detection of SNP and is not indicated in an isolated way for the diagnosis of co-infections.

4 | DISCUSSION

This work demonstrated the first cases of co-infection by classic MYXV and ha-MYXV in one European wild rabbit and one Iberian hare from Tavira, South Portugal. By qPCR, conventional PCR and sequencing analysis, we confirmed the presence of two distinct viruses in a wild rabbit (rabbit-1) and a hare (hare-1). These findings also provide the first detection of a classic MYXV in an Iberian hare, although in co-infection and with low viral load, with the naturally recombinant strain (ha-MYXV).

During a 2018–2019 national surveillance plan conducted within the scope of Project +Coelho 2, 20 leporids collected in Faro district (South Portugal) were analysed for the presence of MYXV, of which eight were European wild rabbits and 12 Iberian hares. None of the rabbits tested positive for myxomatosis, but 67% (8/12) of the Iberian hares were positive for MYXV (ha-MYXV strain). Despite not being detected in the eight rabbits tested from this area in this period, MYXV is endemic all over the country (Duarte et al., 2018, 2021).

Apart from epistaxis and eye bleeding, hare-1 did not show any external or internal lesions, suggesting an acute form of infection. Although the death resulted from trauma, the viral loads found in tissues (Table 3) suggest that viral multiplication had been occurring for a few days.

The bacteriological and parasitological examinations did not show any relevant pathogen that may have contributed to the death of the animals.

Both viral strains were detected in internal organs, confirming systemic distribution. Compared with ha-MYXV, the classic virus was found in lower viral loads in both animals, possibly due to post-infection with classic MYXV or to slower replication. In fact, ha-MXYV appears to have an advantage over classical strains regarding viral multiplication efficiency, leading to a shorter incubation period of the disease and faster killing (Abade dos Santos et al., 2022), in agreement with reports

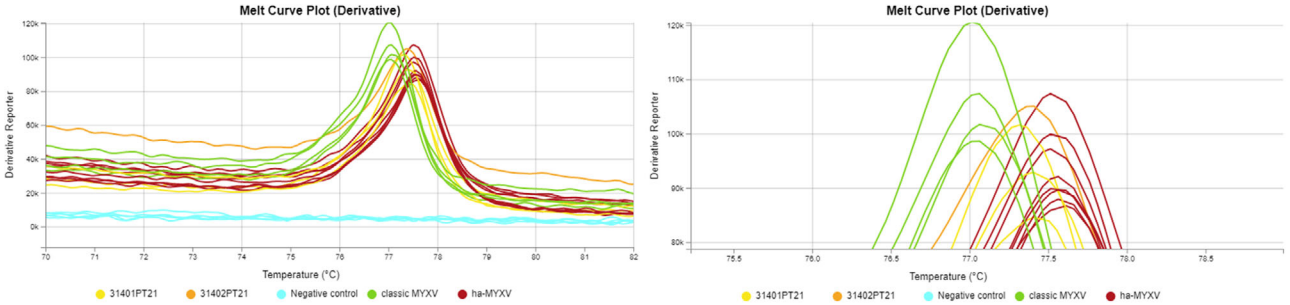

FIGURE 3 (Left) Melt curve plot (derivative) of negative controls (blue curves) classic MYXV strains (green curves), ha-MYXV strains (red curves) and the two co-infected animals 31401PT21 (yellow curves) and 31402PT21 (orange curves). (Right) Higher magnification of the peaks of the three types of samples

of sudden death in hares with myxomatosis (Duarte et al., 2021; García-Bocanegra et al., 2020).

It was not possible to determine which of the two infections occurred first. In the case of the Iberian hare, the infection by ha-MYXV was likely the first, opening space by immunosuppression, to infection by the classical strain of MYXV, taking into account that the classic MYXV does not effectively infect the Iberian hare. However, the detection of another rabbit positive for MYXV in the area may suggest that the sympatry of wild rabbits and hares in this region may have favoured contact and spillover events between rabbits infected with MYXV strains and hares infected with ha-MYXV. European hare cell lines are not permissive to the classical strains of MYXV, and the absence of disease outbreaks in the Iberian hare seems to corroborate that in this species, the classical strains are also non-pathogenic.

In general, co-infections by multiple pathogens may play an important role in deteriorating disease outcomes. Co-infection by strains or variants of the same virus plays a key role in the emergence of new strains and subtypes, such as influenza virus (Myers et al., 2011) and SARS-CoV-2 (Boni et al., 2020). Likewise, the occurrence of MYXV and ha-MYXV co-infections in hares and rabbits may favour events of recombination leading to the generation of new recombinant viruses with distinct biological characteristics.

A high-resolution melting analysis was implemented for the simultaneous detection in the same sample of an M152R sequence containing the cytosine insertion, characteristic of ha-MYXV strains and another sample lacking the cytosine, characteristic of the MYXV strains, to demonstrate the simultaneous presence of the two strains.

The full agreement between the seq9E/seq9F PCR and the *M152R* SNP PCR results, both concordant regarding the presence of ha-MYXV strains in both animals, discarded the remote hypothesis that the presence of the virus without the 2.8 Kbp insertion in the *M009L* gene could have resulted from the loss of the insert during virus multiplication in the host.

To the best of our knowledge, this is the first documented description of co-infection by a classic MYXV and ha-MYXV in a wild rabbit and an Iberian hare. It is crucial to continue monitoring the field strains that circulate in these leporid species to gain a thorough understanding of their evolution and relevance to wild populations. This case represents another threat to the very fragile state of the Iberian hare, particularly due to the ineffectiveness of the use of commercial vaccines (Abade dos Santos et al., 2022).

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ETHICS STATEMENT

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to. No ethical approval was required as no animal was killed for this study.

CONFLICT OF INTEREST

The authors declare no competing interests.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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