UNIVERSIDADE DE LISBOA

FACULDADE DE
MEDICINA VETERINÁRIA

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FACULTAD DE
BIOLOGÍA

IMPACT OF LEPORID VIRAL DISEASES IN IBERIAN ECOSYSTEMS: EMERGENCE,PATHOPHYSIOLOGY, PROPHYLAXIS AND DIAGNOSIS

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Orientadores   Doutora Margarida Dias Duarte

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Thesis specially designed to obtain the degree of Doctor of Veterinary Sciences in the speciality of Animal Health by Faculty of Veterinary Medicine, University of Lisbon and Doctor of Molecular and Cell Biology by Faculty of Biology, University of Oviedo

Júri

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RESUMEN DEL CONTENIDO DE TESIS DOCTORAL

1.- Título de la Tesis	
Español/Otro Idioma: Impacto de las enfermedades virales de los leporidos en los ecosistemas ibéricos: emergencia, fisiopatología, profilaxis y diagnóstico.	Inglés: Impact of leporid viral diseases in Iberian ecosystems: emergence, pathophysiology, prophylaxis and diagnosis

2.- Autor	
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RESUMEN (en español)

El estado alarmante de conservación de las especies de lepóridos en la Península Ibérica justifica la adopción en esta Tesis Doctoral de un enfoque integrador que incluya el estudio de diferentes aspectos de las interfaces virus-huésped-ambiente con respecto a los principales patógenos virales del conejo de monte (Oryctolagus cuniculus algirus) y la liebre ibérica (*Lepus granatensis*) en los ecosistemas mediterráneos.

Los estudios a los que se refiere esta tesis han empleado y puesto a punto metodologías propias de la biología molecular y celular, la genética, la virología, la inmunología y la patología, aplicadas al conejo de monte y a la liebre ibérica, estableciéndose como objetivos de esta Tesis la investigación fisiopatológica de las enfermedades causadas por el virus de la enfermedad hemorrágica del conejo (RHDV2), el virus de la mixomatosis (MYXV) y el leporidae gammaherpesvirus 5 (LeHV-5), su impacto en las especies afectadas, el diseño de nuevos métodos de diagnóstico y la búsqueda de soluciones para mitigar los efectos de estos agentes. Además de reflexionar sobre la interpretación correcta de los diagnósticos moleculares en el contexto de la relación virus-hospedador, como paso previo a la presentación de los trabajos experimentales propiamente dichos, se han puesto a punto algunas metodologías necesarias para su desarrollo, como la extracción de sangre a través de la vena yugular externa en conejos de monte y liebres ibéricas, o procedimientos sencillos para obtener cultivos primarios de fibroblastos de lepóridos.

En esta Tesis se ha demostrado que el tejón euroasiático (*Meles meles*) es susceptible al RHDV2 y que esta especie animal puede actuar como reservorio potencial del virus de la enfermedad hemorragica del conejo, habiendose indentificado la misma cepa de RHDV2 aislada de los tejones en un conejo enano, que tenía una enfermedad prolongada (atípica) y un historial de ineficacia de vacunación con una vacuna comercial para RHDV todavía disponible en el mercado portugués.

Con respecto a la mixomatosis en esta Tesis se recogen los trabajos de detección y análisis de los primeros casos de esta enfermedad en liebres ibéricas asociados a una cepa recombinante natural del virus (ha-MYXV), así como la primera demostración de que el virus recombinante de las liebres puede infectar tanto conejos de monte como domésticos. Adicionalmente se recogen las evidencias de los primeros casos de coinfección del virus recombinante ha-MYXV y el virus mixoma clásico MYXV, tanto en conejo de monte como en liebre ibérica. En un intento de aportar soluciones para el control de la mixomatosis en leporidos silvestres se ha demostrado que las vacunas homólogas comerciales contra la mixomatosis son eficaces en conejos de monte frente a la nueva cepa naturalmente recombinante ha-MYXV pero no protegen frente a la mixomatosis en la liebre ibérica. En estos estudios se ha puesto de manifiesto el potencial protector de una vacuna heteróloga comercial frente a la mixomatosis de las liebres, particularmente cuando se aumenta la dosis vacunal. Para el seguimiento y caracterización de los distintos tipos de virus responsables de la mixomatosis en leporidos se desarrolló un sistema qPCR multiplex que permite una rápida detección y diferenciación entre cepas clásicas de MYXV y los recombinantes naturales ha-MYXV.

En el curso de las investigaciones sobre la mixomatosis en la liebre ibérica se detectó por primera vez en este género la presencia de un herpesvirus, denominado Leporid

gammaherpesvirus 5, para cuya detección se desarrolló y puso a punto un método de diagnóstico basado en qPCR, utilizando sistemas Evagreen o sondas de hidrólisis.

Los diferentes estudios realizados permitieron, en primer lugar, conocer el alarmante estado de conservación de la liebre silvestre en la Península Ibérica por exposición a patógenos endémicos y emergentes, ejemplificado por la emergencia en los últimos tres años de virus de mixomatosis (ha-MYXV) naturalmente recombinante, la detección del mismo virus en conejos de monte y la identificación de un nuevo herpesvirus en la liebre, entre otros agentes de origen bacteriológico y parasitario, que no han sido objeto de esta tesis. En segundo lugar, estos hallazgos nos han permitido comprender cuánto queda por hacer, y saber, en la investigación del estado de las poblaciones silvestres en general, y de lo conejo y liebres en particular. Por último, pero no menos importante, las evidencias que se recogen en esta Tesis apoyan la necesidad de un pleno reconocimiento por parte de la sociedad civil, la clase política y las entidades gubernamentales del valor de estas especies y las gravísimas consecuencias que su estado actual está teniendo en la biodiversidad y mantenimiento del ecosistema mediterráneo.

RESUMEN (en Inglés)

The alarming state of conservation of leporid species in the Iberian Peninsula justifies the adoption in this Doctoral Thesis of an integrative approach that includes the study of different aspects of the virus-host-environment interfaces with respect to the main viral pathogens of the wild rabbit (*Oryctolagus cuniculus algirus*) and the Iberian hare (*Lepus granatensis*) in Mediterranean ecosystems.

The studies to which this thesis refers to have used and developed methodologies of molecular and cellular biology, genetics, virology, immunology and pathology, applied to the wild rabbit and the Iberian hare, establishing as objectives of this Thesis the pathophysiological research of the diseases caused by rabbit hemorrhagic disease virus (RHDV2), myxoma virus (MYXV) and leporidae gammaherpesvirus 5 (LeHV-5), their impact on affected species, the design of new diagnostic methods and the search for control measures to mitigate the effects of these agents.

In addition to reflecting on the correct interpretation of molecular diagnoses in the context of the virus-host relationship, as a previous step to the presentation of the experimental results, some methodologies have been developed which were necessary for their implementation, such as the extraction of blood through the external jugular vein in wild rabbits and Iberian hares, or simple procedures in order to obtain primary cultures from leporid fibroblasts.

In this thesis it has been shown that the Eurasian badger (*Meles meles*) is susceptible to RHDV2 and that this species can act as a potential reservoir of the rabbit hemorrhagic disease virus. It was also shown that the same RHDV2 strain isolated from badgers could infect a dwarf rabbit, who had a prolonged (atypical) illness and a history of vaccination ineffectiveness with a commercial RHDV vaccine still available on the Portuguese market.

With regard to myxomatosis, this thesis includes the detection and analysis of the first cases of this disease in Iberian hares, associated with a natural recombinant strain of the virus (ha-MYXV), as well as the first demonstration that the recombinant virus of hares can infect both wild and domestic rabbits. Additionally, evidence of the first cases of co-infection of the recombinant virus ha-MYXV and the classic myxoma virus MYXV is reported, both in wild rabbits and in Iberian hares. In an attempt to provide solutions for the control of myxomatosis in wild leporids, it has been shown that commercial homologous vaccines against myxomatosis are effective in wild rabbits against the new naturally recombinant strain ha-MYXV, but failed to protect the Iberian hare against myxomatosis. These studies have highlighted the protective potential against hare's myxomatosis of a commercial heterologous vaccine, particularly when the inocculated dose is increased. For monitoring and characterization of the different types of viruses responsible for myxomatosis in leporids, a multiplex qPCR system

has been developed that allows for a rapid detection and differentiation between classic strains of MYXV and the natural recombinants ha-MYXV.

In the course of research on myxomatosis in the Iberian hare, the presence of a herpesvirus,

called Leporid gammaherpesvirus 5, was detected for the first time in this genus, for whose detection a diagnostic method based on qPCR was developed and standardized, using Evagreen systems or hydrolysis probes.

The different studies carried out allowed, first of all, to realize the alarming conservation state of the wild hare in the Iberian Peninsula due to exposure to endemic and emerging pathogens, exemplified by the emergence, at least in the last three years, of naturally recombinant myxomatosis virus (ha-MYXV), also detected in wild rabbits, and the identification of a new herpesvirus in a hare, amongst other agents of bacteriological and parasitic origin, which were not the subject of this thesis. Secondly, these findings have allowed us to understand how much remains to be investigated regarding the wild animal populations in general, and the European rabbits and hares in particular. Finally, the evidence collected in this thesis supports the need for full recognition by the society in general and by the political and government entities of the value of these species for the biodiversity and the maintenance of the Mediterranean ecosystem and the very serious damages caused by their current situation.

SR. PRESIDENTE DE LA COMISIÓN ACADÉMICA DEL PROGRAMA DE DOCTORADO EN BIOLOGÍA MOLECULAR Y CELULAR

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Olha à tua volta, vê quão pequeno és, e viverás melhor
Olha para trás, lembra o teu caminho, e saberás para onde ir

Look around you, see how small you are, and you will live better
Look back, remember your path, and you'll know where to go

Fábio A. Abade dos Santos, 2021

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Não é certamente com um pensamento vertical que escrevo as frases seguintes. Este trabalho é de muitos, os muitos que apoiaram e acompanharam esta jornada de três anos, que envolveu lágrimas e sorrisos, mais dos últimos.

Aos meus pais e irmãos não lhes devo este trabalho, mas antes tudo e tanto, que seria injusto e impossível aqui tentar elencar. À minha restante família, em particular à memória do meu avô António, com quem tive o privilégio de privar a minha infância e da qual transporto as melhores memórias, e de quem transporto o dom, amor e respeito incondicionais pelos animais.

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À Professora Conceição Peleteiro que me introduziu à investigação. Será sempre uma referência e um exemplo para mim, porque sem ela a minha carreira teria sido, muito provavelmente, diferente, e certamente não tão boa e divertida. Ensinou-me a ver o mundo a rosa e azul-púrpura e que mundos bonitos que já vimos juntos.

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reposicionada à escala da vida.

Aos meus amigos (não vou referir nomes com receio de me esquecer-me de algum), "científicos", "da escola", "de curso", "de infância", "e afins", devo-lhes as ferramentas da personalidade, da inteligência emocional, as vivências, aprendizagem e o espírito que certamente permitiram pegar na pedra, parti-la, fazer cimento, por areia na proporção 1:3, cozer tijolo e erguer esta tese.

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RESUMO

O estado alarmante de conservação das espécies de leporídeos na Península Ibérica justifica a adopção nesta Tese de Doutoramento de uma abordagem integrativa que inclui o estudo de diferentes aspectos das interfaces vírus-hospedeiro-ambiente relativos aos principais agentes patogénicos virais do coelho-bravo (*Oryctolagus cuniculus algirus*) e da lebre ibérica (*Lepus granatensis*) nos ecossistemas mediterrânicos.

Os estudos referidos nesta tese aplicaram e refinaram metodologias de biologia molecular e celular, genética, virologia, imunologia e patologia, aplicadas ao coelho-bravo e à lebre ibérica, estabelecendo-se como objectivos desta tese a investigação fisiopatológica da doenças causadas pelo vírus da doença hemorrágica do coelho (RHDV2), pelo vírus da mixomatose (MYXV) e pelo leporid gammaherpesvirus 5 (LeHV-5), os seus impactos nas espécies afetadas, o desenho de novos métodos de diagnóstico e a procura de soluções para mitigar os efeitos desses agentes nas populações selvagens de leporideos.

Além de refletir sobre a correta interpretação dos diagnósticos moleculares no contexto da relação vírus-hospedeiro, como etapa preliminar à apresentação do próprio trabalho experimental, foram desenvolvidas e implementadas algumas metodologias necessárias ao seu desenvolvimento dos estudos subsequentes, como a extração de sangue através da veia jugular externa em coelhos selvagens e lebres ibéricas, ou procedimentos simples para obtenção de culturas primárias de fibroblastos de leporídeos.

Nesta Tese foi demonstrado que o texugo euro-asiático (*Meles meles*) é suscetível ao RHDV2 e que esta espécie animal pode atuar como um potencial reservatório do vírus da doença hemorrágica do coelho. A mesma estirpe de RHDV2 isolada de texugos foi identificada num coelho anão que apresentava doença prolongada (atípica) e histórico de falha vacinal com uma vacina comercial RHDV, na altura disponível no mercado português.

No que diz respeito à mixomatose, esta Tese inclui a detecção e análise dos primeiros casos desta doença em lebres ibéricas, associada a uma estirpe naturalmente recombinante do vírus (ha-MYXV), bem como a primeira demonstração de que este vírus recombinante de lebres pode também infectar coelhos selvagens e domésticos. Adicionalmente, foram recolhidas evidências dos primeiros casos de co-infecção do vírus recombinante ha-MYXV e do vírus do mixoma clássico MYXV, tanto em coelho selvagem como em lebre ibérica. Na tentativa de fornecer soluções para o controlo da mixomatose em leporídeos selvagens, foi demonstrado que as vacinas homólogas comerciais contra a mixomatose são eficazes em coelhos selvagens contra a nova estirpe naturalmente recombinante ha-MYXV mas não protegem contra a mixomatose na lebre. Esses estudos revelaram o potencial protetor de uma vacina heteróloga comercial contra a mixomatose em lebres, principalmente quando a dose da vacina é aumentada. Para a monitorização e caracterização dos diferentes tipos de

vírus responsáveis pela mixomatose em leporídeos, foi desenvolvido um sistema de qPCR multiplex que permite a rápida deteção e diferenciação entre estirpes clássicas de MYXV e ha-MYXV, recombinante natural.

No decurso da investigação sobre mixomatose na lebre ibérica, foi detectada, pela primeira vez neste género, a presença de um herpesvírus, denominado Leporid gammaherpesvirus 5, para cuja detecção foi desenvolvido e validado um método de diagnóstico baseado em qPCR, utilizando sistemas Evagreen ou sondas de hidrólise.

Os diferentes estudos realizados permitiram, em primeiro lugar, reconhecer o estado alarmante de conservação da lebre-ibérica na Península Ibérica devido à exposição a múltiplos agentes patogénicos endémicos e emergentes, exemplificado pela emergência nos últimos três anos do vírus da mixomatose (ha-MYXV) naturalmente recombinante, pela detecção do mesmo vírus em coelhos selvagens e pela identificação de um novo herpesvírus em lebre, entre outros agentes de origem bacteriológica e parasitária, que não foram objeto desta tese. Em segundo lugar, essas descobertas permitiram entender o quanto ainda há a ser investigado na caracterização do estado das populações selvagens em geral e do coelho-europeu e das lebres em particular. Por último, mas não menos importante, as evidências recolhidas nesta Tese corroboram a necessidade do pleno reconhecimento por parte da sociedade civil, classe política e entidades governamentais, do valor destas espécies e das gravíssimas consequências que o seu estado atual está a ter na biodiversidade e na manutenção das espécies do ecossistema mediterrâneo.

Palavras-chave: *Oryctolagus cuniculus algirus*, *Lepus granatensis*, mixomatose, doença hemorrágica viral, herpesvirus

RESUMEN

El estado alarmante de conservación de las especies de lepóridos en la Península Ibérica justifica la adopción en esta Tesis Doctoral de un enfoque integrador que incluya el estudio de diferentes aspectos de las interfaces virus-huésped-ambiente con respecto a los principales patógenos virales del conejo de monte (*Oryctolagus cuniculus algirus*) y la liebre ibérica (*Lepus granatensis*) en los ecosistemas mediterráneos.

Los estudios a los que se refiere esta tesis han empleado y puesto a punto metodologías propias de la biología molecular y celular, la genética, la virología, la inmunología y la patología, aplicadas al conejo de monte y a la liebre ibérica, estableciéndose como objetivos de esta Tesis la investigación fisiopatológica de las enfermedades causadas por el virus de la enfermedad hemorrágica del conejo (RHDV2), el virus de la mixomatosis (MYXV) y el leporidae gammaherpesvirus 5 (LeHV-5), su impacto en las especies afectadas, el diseño de nuevos métodos de diagnóstico y la búsqueda de soluciones para mitigar los efectos de estos agentes.

Además de reflexionar sobre la interpretación correcta de los diagnósticos moleculares en el contexto de la relación virus-hospedador, como paso previo a la presentación de los trabajos experimentales propiamente dichos, se han puesto a punto algunas metodologías necesarias para su desarrollo, como la extracción de sangre a través de la vena yugular externa en conejos de monte y liebres ibéricas, o procedimientos sencillos para obtener cultivos primarios de fibroblastos de lepóridos.

En esta Tesis se ha demostrado que el tejón euroasiático (*Meles meles*) es susceptible al RHDV2 y que esta especie animal puede actuar como reservorio potencial del virus de la enfermedad hemorragica del conejo, habiendose indentificado la misma cepa de RHDV2 aislada de los tejones en un conejo enano, que tenía una enfermedad prolongada (atípica) y un historial de ineficacia de vacunación con una vacuna comercial para RHDV todavía disponible en el mercado portugués.

Con respecto a la mixomatosis en esta Tesis se recogen los trabajos de detección y análisis de los primeros casos de esta enfermedad en liebres ibéricas asociados a una cepa recombinante natural del virus (ha-MYXV), así como la primera demostración de que el virus recombinante de las liebres puede infectar tanto conejos de monte como domésticos. Adicionalmente se recogen las evidencias de los primeros casos de coinfección del virus recombinante ha-MYXV y el virus mixoma clásico MYXV, tanto en conejo de monte como en liebre ibérica. En un intento de aportar soluciones para el control de la mixomatosis en leporidos silvestres se ha demostrado que las vacunas homólogas comerciales contra la mixomatosis son eficaces en conejos de monte frente a la nueva cepa naturalmente recombinante ha-MYXV pero no protegen frente a la mixomatosis en la liebre ibérica. En

estos estudios se ha puesto de manifiesto el potencial protector de una vacuna heteróloga comercial frente a la mixomatosis de las liebres, particularmente cuando se aumenta la dosis vacunal. Para el seguimiento y caracterización de los distintos tipos de virus responsables de la mixomatosis en leporidos se desarrolló un sistema qPCR multiplex que permite una rápida detección y diferenciación entre cepas clásicas de MYXV y los recombinantes naturales ha-MYXV.

En el curso de las investigaciones sobre la mixomatosis en la liebre ibérica se detectó por primera vez en este género la presencia de un herpesvirus, denominado Leporid gammaherpesvirus 5, para cuya detección se desarrolló y puso a punto un método de diagnóstico basado en qPCR, utilizando sistemas Evagreen o sondas de hidrólisis.

Los diferentes estudios realizados permitieron, en primer lugar, conocer el alarmante estado de conservación de la liebre silvestre en la Península Ibérica por exposición a patógenos endémicos y emergentes, ejemplificado por la emergencia en los últimos tres años de virus de mixomatosis (ha-MYXV) naturalmente recombinante, la detección del mismo virus en conejos de monte y la identificación de un nuevo herpesvirus en la liebre, entre otros agentes de origen bacteriológico y parasitario, que no han sido objeto de esta tesis. En segundo lugar, estos hallazgos nos han permitido comprender cuánto queda por hacer, y saber, en la investigación del estado de las poblaciones silvestres en general, y de lo conejo y liebres en particular. Por último, pero no menos importante, las evidencias que se recogen en esta Tesis apoyan la necesidad de un pleno reconocimiento por parte de la sociedad civil, la clase política y las entidades gubernamentales del valor de estas especies y las gravísimas consecuencias que su estado actual está teniendo en la biodiversidad y mantenimiento del ecosistema mediterráneo.

Palabras-clave: *Oryctolagus cuniculus algirus*, *Lepus granatensis*, mixomatosis, enfermedad hemorrágica del conejo, herpesvirus

ABSTRACT

The alarming state of conservation of leporid species in the Iberian Peninsula justifies the adoption in this Doctoral Thesis of an integrative approach that includes the study of different aspects of the virus-host-environment interfaces with respect to the main viral pathogens of the wild rabbit (*Oryctolagus cuniculus algirus*) and the Iberian hare (*Lepus granatensis*) in Mediterranean ecosystems.

The studies to which this thesis refers to have used and developed methodologies of molecular and cellular biology, genetics, virology, immunology and pathology, applied to the wild rabbit and the Iberian hare, establishing as objectives of this Thesis the pathophysiological research of the diseases caused by rabbit hemorrhagic disease virus (RHDV2), myxoma virus (MYXV) and leporidae gammaherpesvirus 5 (LeHV-5), their impact on affected species, the design of new diagnostic methods and the search for control measures to mitigate the effects of these agents.

In addition to reflecting on the correct interpretation of molecular diagnoses in the context of the virus-host relationship, as a previous step to the presentation of the experimental results, some methodologies have been developed which were necessary for their implementation, such as the extraction of blood through the external jugular vein in wild rabbits and Iberian hares, or simple procedures in order to obtain primary cultures from leporid fibroblasts.

In this thesis it has been shown that the Eurasian badger (*Meles meles*) is susceptible to RHDV2 and that this species can act as a potential reservoir of the rabbit hemorrhagic disease virus. It was also shown that the same RHDV2 strain isolated from badgers could infect a dwarf rabbit, who had a prolonged (atypical) illness and a history of vaccination ineffectiveness with a commercial RHDV vaccine still available on the Portuguese market.

With regard to myxomatosis, this thesis includes the detection and analysis of the first cases of this disease in Iberian hares, associated with a natural recombinant strain of the virus (ha-MYXV), as well as the first demonstration that the recombinant virus of hares can infect both wild and domestic rabbits. Additionally, evidence of the first cases of co-infection of the recombinant virus ha-MYXV and the classic myxoma virus MYXV is reported, both in wild rabbits and in Iberian hares. In an attempt to provide solutions for the control of myxomatosis in wild leporids, it has been shown that commercial homologous vaccines against myxomatosis are effective in wild rabbits against the new naturally recombinant strain ha-MYXV, but failed to protect the Iberian hare against myxomatosis. These studies have highlighted the protective potential against hare's myxomatosis of a commercial heterologous vaccine, particularly when the inoculated dose is increased. For monitoring and characterization of the different types of viruses responsible for myxomatosis in leporids, a multiplex qPCR system

has been developed that allows for a rapid detection and differentiation between classic strains of MYXV and the natural recombinants ha-MYXV.

In the course of research on myxomatosis in the Iberian hare, the presence of a herpesvirus, called Leporid gammaherpesvirus 5, was detected for the first time in this genus, for whose detection a diagnostic method based on qPCR was developed and standardized, using Evagreen systems or hydrolysis probes.

The different studies carried out allowed, first of all, to realize the alarming conservation state of the wild hare in the Iberian Peninsula due to exposure to endemic and emerging pathogens, exemplified by the emergence, at least in the last three years, of naturally recombinant myxomatosis virus (ha-MYXV), also detected in wild rabbits, and the identification of a new herpesvirus in a hare, amongst other agents of bacteriological and parasitic origin, which were not the subject of this thesis. Secondly, these findings have allowed us to understand how much remains to be investigated regarding the wild animal populations in general, and the European rabbits and hares in particular. Finally, the evidence collected in this thesis supports the need for full recognition by the society in general and by the political and government entities of the value of these species for the biodiversity and the maintenance of the Mediterranean ecosystem and the very serious damages caused by their current situation.

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LIST OF ABREVIATIONS

AC	After collection
AGID	Agar gel Immunodiffusion
ASA	American Society of Anaesthesiology criteria
AST	Average Survival Time
BC	Before collection
Bpm	Breaths per minute
BS	Before sedation
CFR	Case Fatality Rates
CFT	Complement Fixation Test
CPE	Cytopathic effect or cytopathogenic effect
DFA	Direct Fluorescent Antibody
dFT	direct Immunofluorescence Test
DPOL	DNA polymerase
e.g.	exempli gratia
EBHSV	European Brown Hare Syndrome Virus
ECG	Eletrocardiogram
EJV	External Jugular Vein
ELISA	Enzyme-Linked Immunosorbent Assay
EM	Electron microscopy
FAT	direct immunofluorescence test
gRNA	Genomic Ribonucleic acid
HA	Hemagglutination
HaCV	Hare Calicivirus
ID50	50% infective dose
IEM	Immunoelectron microscopy
IFAT	indirect immunofluorescence test
iFT	indirect Fuorescence Test
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IPMA	immunoperoxidase monolayer assay
IUCN	International Union for Conservation of Nature
Kb	Kilobases
LAMP	Loop-mediated isothermal amplification
LeHV-5	Leporid gammaherpesvirus 5
LNA	locked nucleic acids
MAbs	Monoclonal Antibodies
MYXV	Myxoma virus
nsEM	Negative-staining electron microscopy
O.c.algirus	*Oryctolagus cuniculus algirus*
O.c.cuniculus	*Oryctolagus cuniculus cuniculus*
OIE	World Organisation for Animal Health
ORF	Open Reading Frame
PBS	Phosphate-Buffered Saline

PCR	Polymerase Chain Reaction
PCR-RFLPs	polymerase chain reaction - restriction fragment length polymorphism
pfu	plaque-forming unit
RCV	Rabbit Calicivirus
RdRp	RNA dependent RNA polymerase
RHD	Rabbit haemorrhagic disease
RHDV	Rabbit haemorrhagic disease virus
RHDV2	Rabbit haemorrhagic disease type 2
SD	Standard Deviation
SDS-PAGE	Sodium dodecyl-sulfate polyacrylamide gel electrophoresis
SFV	Shope Fibroma Virus
sgRNA	Subgenomic Ribonucleic acid
SLS	Standard Laboratory Strain
Th1	Th1 helper cells
Th2	Th2 helper cells
TIRs	Terminal Inverted Repeats
VLPs	Viral-like particles
VP10	Viral Protein 10 (minor capsid protein)
VP60	Viral Protein 60 (major capsid protein)

Chapter 1

Introduction and Research objectives

■ ■

Chapter 1 reviews the importance of the mediterranean ecosystems, including the role of the Iberian leporids in the trophic network and their conservation status. It also provides a bibliographic review of the main viruses that affect Leporids, namely reviewing their emergence and spatiotemporal distribution, pathophysiology, prohylaxis and diagnosis.

■ ■
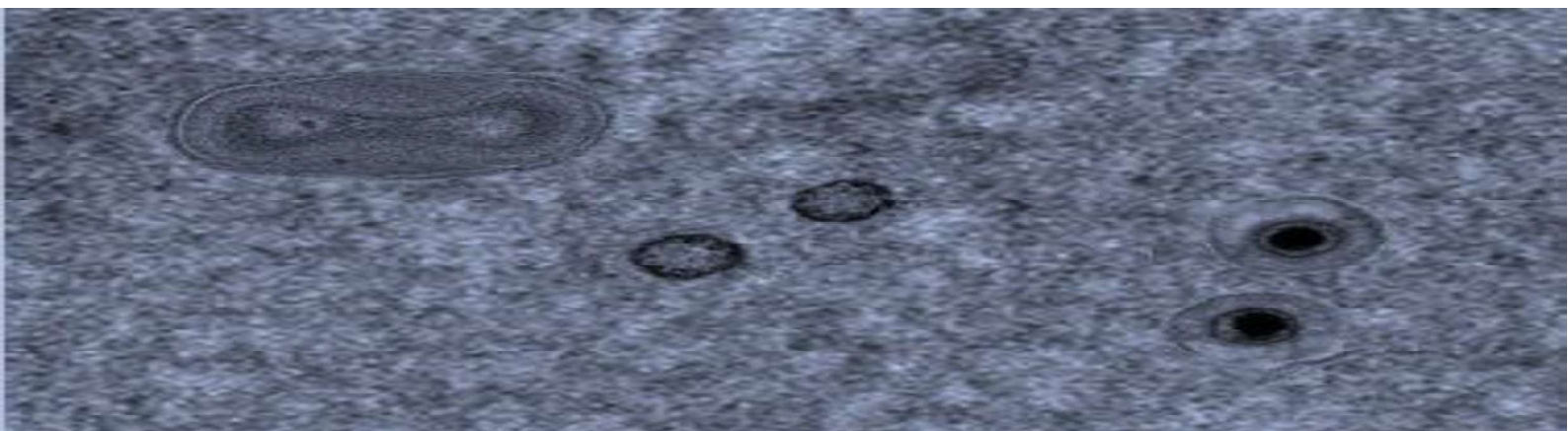
1.1. Mediterranean Basin hotspot

Planet Earth holds around 34 global diversity hotspots - areas featuring exceptional concentrations of endemic species - whose value is uncountable and indisputable (Myers et al. 2000; Mittermeier et al. 2005) - that are experiencing exceptional loss of habitat. In these hotspots, the decline or disappearance of key species has been identified as the main threat, leading to catastrophic cascading effects on the whole system (Stuart Chapin et al. 2000). The western corner of the Mediterranean Basin hotspot is a beautiful and irreplaceable piece of all global taxonomic diversity, second only to the tropics in importance (Cowling et al. 1996), containing the same plant richness (30,000 taxa) of all tropical Africa (four times larger) and 10.8 species/1000 km^2, higher than China, Zaira, India, Brazil and lower than Colombia or Panama (with 40 to 90 species /1000 km^2) (Médail and Quézel 1999).

1.2. Particularities of Iberian Leporids - hotspot engineers

The wild rabbit (*Oryctolagus cuniculus*, Linnaeus, 1758) and the Iberian hare (*Lepus granatensis*, Rosenhauer, 1856) are the only two species of wild leporids found in Portugal. The wild rabbit is originated from the Iberian Peninsula, where two subspecies (*Oryctolagus cuniculus algirus* Loche, 1858 and *Oryctolagus cuniculus cuniculus* Linnaeus, 1758) are described, which diverged about two million years ago and are currently different in terms of genetics, morphology, and behavioural ecology. The natural distribution of the subspecies *O. c. algirus* is restricted to the southwest of the Iberian Peninsula and the geographical distribution pattern is well delimited (Ferrand 1995; Branco et al. 2000; Geraldes et al. 2006). The subspecies *O. c. algirus* is the unique present in Portugal, with adults weighing an average of 1.100 Kg with a maximum of 1.400 Kg (Soriguer 1980; Ferreira and Ferreira 2014), also present in Spain, North Africa and Atlantic islands (Madeira, Azores and Canaries). However, these two subspecies are present in a hybrid zone that crosses the Iberian peninsula between Almeria (Spain) and Santiago (Spain), passing through Bragança (Portugal) (Branco et al. 2000; Alda and Doadrio 2014).

The Iberian hare (*Lepus granatensis* Rosenhauer, 1856) is also endemic and indigenous to the Iberian Peninsula, and its geographical area extends from Portugal across southwest Spain. In Spain, another two species can be found, the Broom hare (*Lepus castroviejoi* Palacios, 1976) and the European brown hare (*Lepus europaeus* Pallas, 1778).

European rabbit (*Oryctolagus cuniculus*), the sole representative of its genus and one of the rare mammals originally domesticated in Western Europe, is endemic from the Iberian

Peninsula, having been widely distributed from Iberia and France in the late Pleistocene and to Europe, South America, Australia and some oceanic islands (Gibb 1990; Monnerot et al. 1994) by anthropogenic effect. This species is present in diverse ecosystem types, from Mediterranean scrublands to agroecosystems, mountainous areas, as well as coastal and dunes (Dellafiore et al. 2014).

European wild rabbit (*Oryctolagus cuniculus*) and hare (*Lepus* granatensis) populations are currently declining to alarming levels (Ward 2005; Duarte et al. 2020), representing a huge concern for the continuity of the species themselves, and all the trophic chains involved and respective ecosystems. The European rabbit is an opportunistic and highly adaptable species, so its current fragile state (plague in the past and threatened of extinction nowadays) should be a major topic of concern and discussion, given the implications of the wild rabbit decline on many other less resilient species.

The rabbit is considered a successful colonizer with a worldwide distribution, from subtropical to sub-Antarctic climates, being present in Australia and New Zealand, South America, and in more than 800 islands throughout the world (Flux and Fullagar 1992; Thompson and King 1994)

Wild rabbit is the principal multifunctional ecosystem engineer, impacting in vegetation structure and composition, in the herbivory and seed dispersal, in soil condition, with indirect impact in the population of the invertebrates namely as diggers, and finally, together with Iberian hare, as key preys for more than 40 species of predators (Delibes-Mateos et al. 2008). Ecologically, the hare species have great importance namely because hares are the prey of several species like the golden eagle (*Aquila chrysaetos*), European wildcat (*Felis silvestris*), fox (*Vulpes vulpes*), Eurasian eagle-owl (*Bubo bubo*) and others. Rabbit disappearance in this ecosystem would precipitate further species extinctions (Ferreira 2012). Large predators elect leporids as principal prey, while smaller predators choose leporids as alternative prey (Delibes and Hiraldo 1981). Rabbits are so important and iconic for the Mediterranean scrubland of southwestern Europe, that some ecologists name it "the rabbit's ecosystem" (Delibes-Mateos et al. 2008). Grazing by rabbits and hares prevents tall plant species from becoming dominant, leading to mosaics of open areas promoting the growth of a herbaceous layer in scrubland. This mosaic, typic of Mediterranean ecosystems, promotes the ecosystem stability and the maintenance of several species of plants, vertebrates and invertebrates, increasing habitat complexity and heterogeneity (Gómez Sal et al. 1999; Van der Wal et al. 2000; Delibes-Mateos et al. 2008). This mosaic system is particularly important in the Iberian Peninsula, where the small mammal community is richer than in other Mediterranean areas (Cagnin et al. 1998). Besides their ecological role, the wild rabbit and Iberian hare are important game species economically and socially, namely in Portugal and Spain, but also in

other countries.

1.3. Leporids' populations trend

Before the second half of the XXth century, the wild rabbit was abundant in the Iberian peninsula (Ferreira and Delibes-Mateos 2010; Delibes-Mateos et al. 2014). Since then, it has been showing a negative trend, mainly due to habitat loss and fragmentation conducing to changes in the Mediterranean mosaic that characterize the traditional Iberian agricultural landscape, and the emergence of viral diseases (Delibes-Mateos et al. 2009). These factors culminated in the recent IUCN reclassification of the conservation status to "danger of extinction" (Villafuerte and Delibes-Mateos 2019).

The rabbit is a paradigmatic example of a wild species with spatiotemporal fluctuations that leads to conflicts between conservationists, farmers and hunters. The spatial distribution of wild rabbit is so heterogeneous that is possible to consider it a "pest" or "locally extinct" just a few kilometres apart (Ferreira and Delibes-Mateos 2010). Another paradigmatic particularity of this species is that despite currently being considered "endangered of extinction" by the IUCNF since 2019, it is also one of the most important small-game species in the Iberia.

1.4. Leporids' threats of viral aetiology

The International Conservation Union (IUCN) in the Red Book of Threatened Species (2019), attributed the status of "endangered", for the first time in history to *Oryctolagus cuniculus* and, the status of "minor concern" to *Lepus granatensis*, although the declining trend of this last species was recognized (Duarte et al. 2020).

The anthropogenic effects on the ecosystems are global and there is no longer any place on the planet that is not affected by human activity, either directly or indirectly (Boyle et al. 2007). The introduction, negligent or intentional, of foreign species or microorganisms in ecosystems where they do not belong, is a human-induced change that has a potentially devastating impact (Vitousek et al. 2015).

Myxomatosis is an example of the devastating effects, caused by a terrible disease introduced intentionally by human hands in France in the 1950s, having been responsible for a significant decline and mortality of wild rabbit populations (Armour and Thompson 1954). Three decades later, the viral haemorrhagic disease caused by a calicivirus, emerged. Until the 1950s, the population density of wild rabbits was very high in Portugal and Spain, even

leading to agricultural losses and the occasional description of rabbits as a "plague". Globalization increased the connectivity between ecosystems, contributing to the emergence or re-emergence of pathogens (Tatem et al. 2006). However, in the last decades, wild rabbit populations have suffered a sharp decrease, both in number and in geographic distribution, is currently estimated to be reduced to 5 to 10% of the population existing 50 years ago and, even so, still decreasing at a rate of about 20% per year in the Iberian Peninsula (Delibes-Mateos et al. 2008; Monterroso et al. 2016). Despite its importance being recognized for a long time, the decrease in populations of wild rabbit and Iberian hare has been occurring continuously for several decades. This scenario has intensified particularly since 2011, with the emergence of a new virus - Rabbit haemorrhagic disease virus type 2 - able to escape the existing vaccines against RHDV and showing a distinct antigenic and pathologic profile (Dalton et al. 2012).

Myxoma virus (MYXV) and rabbit haemorrhagic disease virus (RHDV / RHDV2) are the major pathogen threats for the European rabbit (*Oryctolagus cuniculus*), and may occasionally be found simultaneously (Carvalho et al., 2020; Duarte et al., 2018). These two diseases altered the equilibrium of the Iberian Mediterranean ecosystem (Delibes-Mateos et al. 2008; Delibes-Mateos et al. 2009).

Other viruses have been described in wild leporids, such as herpesviruses: Leporid herpesvirus 1 (LHV-1), Leporid herpesvirus 2 (LHV-2), Leporid herpesvirus 3 (LHV-3), Leporid herpesvirus 4 (LHV-4) and Leporid gammaherpesvirus 5 (LeHV-5). The most common naturally occurring herpesvirus infections identified in rabbits are LHV-2 and LHV-3 (revised by Jin et al., 2008), which alongside LHV-1 belong to the *Gammaherpesvirinae* subfamily. The LHV-4 is an alphaherpesvirus and is more pathogenic, causing fatal infections. The recently described LeHV-5 is the unique herpesvirus described in *Lepus* genus, having been associated with skin and external genitalia severe pathology (Abade dos Santos et al., 2020).

1.5. Lagoviruses

1.5.1. Emergence and spatiotemporal distribution

The International Committee on Taxonomy of Viruses (ICTV) currently recognizes within the *Caliciviridae* family 11 genera of viruses, namely: *Bavovirus, Lagovirus, Minovirus, Nacovirus, Nebovirus, Norovirus, Recovirus, Salovirus, Sapovirus, Valovirus* and *Vesivirus* (Vinjé et al. 2019).

The rabbit hemorrhagic disease virus (RHDV) is the etiological agent of the Rabbit

hemorrhagic disease (that affects both subspecies of the European rabbit), so called given the severe dysregulation of the coagulation system. It is a single-stranded, positive-sense RNA virus, belonging to *Lagovirus* genus, family *Caliciviridae*. This genus also includes the European brown hare syndrome virus (EBHSV), and other non-pathogenic viruses, the rabbit caliciviruses (RCVs) or hare caliciviruses (HCVs) (ICTV 2021).

The discrimination between RHDV and EBHSV was possible by immunoelectron microscopy (Chasey et al. 1992), hemagglutination (Nowotny et al. 1990), and ELISA (Capucci et al. 1991), showing that the two viruses represent two distinct entities.

Rabbit haemorrhagic disease integrates the OIE list of notifiable terrestrial and aquatic animal diseases, which in 2016 replaced the former Lists A and B (OIE). The former List A comprised transmissible diseases with potential for very serious and rapid spread, irrespective of national borders, serious socio-economic or public health consequences and of major importance in the international trade of animals and animal products (OIE). The previous List B, included transmissible diseases considered to be of socio-economic and/or public health importance within countries and significant in the international trade of animals and animal products. RHD integrated List B in the past.

Lagoviridae includes positive-sense single-stranded RNA viruses with a non-enveloped icosahedral capsid and a spherical morphology (Ohlinger et al. 1990; Parra and Prieto 1990; Meyers et al. 1991). The major capsid protein VP1/VP60 forms the structure of the virion and the minor structural protein VP2/VP10 is responsible for stability after the incorporation of viral RNA, covalently linked to the VPg (viral protein genome-linked), which is essential for replication (ICTV 2021).

RHDV genome is around 7.4kb (precisely 7437 nucleotides long) and is divided into two ORFs, ORF1 encoding a polyprotein that is cleaved into several non-structural proteins and the major structural capsid protein, VP60 or VP1 (60 KDa), and ORF2 that encodes the minor structural protein called VP10 or VP2 (Ohlinger et al. 1990; Wirblich et al. 1996).

In addition to the genomic RNA (gRNA), the virions contain a subgenomic mRNA (sgRNA) with 2.2 kb, which is collinear with the 3' end of the genomic RNA (Meyers et al. 1991; Wirblich et al. 1996). sgRNA is packaged together with the genomic RNA. Both the genomic and subgenomic RNA are polyadenylated in the 3' end, and covalently linked to a protein (VPg, viral genome-linked) in its 5' end (Meyers et al. 1991; Machín et al. 2001).

In electron microscopy, a high percentage of viral particles present an electron-dense core with an approximate diameter of 23-25 nm, delineated by a rim from which radiate ten short (5 to 6 nm long) peripherical projections, regularly distributed (Capucci et al. 1991; Sibilia et al. 1995)

The viral capsid comprises 90 arch-like dimers of the capsid protein, forming 32 cup-shaped

depressions (*Calix* in Latin for cup or chalice originating from the family *Caliciviridae* name) arranged in a T = 3 icosahedral symmetry (Valicek et al. 1990; Thouvenin et al. 1997; Bárcena et al. 2004; Luque et al. 2012)

Each protein monomer consists of a shell domain (S) forming an icosahedral shell, with an arm at the N-terminal region (the N-terminal arm or NTA), fronting the inner surface of the capsid shell and connected by a hinge (H) to a flexible protruding domain (P) that encompasses the C-terminal region forming a protrusion emanating from the shell. This P domain is subdivided into the subdomains P1 (the stem or leg of the protrusion) and P2 (top of the protrusion) (Bárcena et al. 2004; Hu et al. 2010; Luque et al. 2012; Bárcena et al. 2015). While the VP60 N-terminal region is buried within the particle, the C-terminal region is exposed on its surface. The S domain is well conserved while the P domain contains determinants for virus-host receptor interactions and antigenic diversity (Bárcena et al. 2004; Hu et al. 2010; Luque et al. 2012; Bárcena et al. 2015). Of the two P1 and P2 subdomains, the last is the most variable and located at the outermost surface region of the viral capsid protein (Bárcena et al. 2015).

In the VP60 the main viral antigen, the exposed surface loop—L1 (a.a. 300–318) from the P2 subdomain exhibit a higher variability between the strains and contains neutralizing antibody inducing epitopes (Capucci et al. 1995; Capucci et al. 1998; Schirrmeier et al. 1999). Despite inducing high titers of virus-neutralizing antibodies, the cross-protective immunity between the different genotypes is very limited (Capucci et al. 1995; Capucci et al. 1998; Schirrmeier et al. 1999; Kinnear and Linde 2010; Dalton et al. 2014; Calvete, Mendoza, M.. Sarto, et al. 2019). Given the high variability of VP60 and the frequency of recombination, it is predictable that new RHDV genogroups will emerge in the future.

The viral protein VP10 is a basic minor structural protein with 12.7 kDa present in small amounts (Wirblich et al. 1996; Meyers et al. 2000; Liu et al. 2008; Chen et al. 2009), being expressed approximately at 20% of the level of the VP60 (Meyers and Tu 2003). This protein is conserved in all caliciviruses, suggesting an important role in the virus cycle namely in the release of virions from the infected cells (Wirblich et al. 1996; Liu et al. 2008; Chen et al. 2009).

The *Lagovirus* genome, similarly to *Sapovirus* and *Nebovirus,* has a genome organized into two major ORF while the *Norovirus*, *Vesivirus* and *Recovirus* (all from *Caliciviridae* family) are organized in three ORF (reviewed in (Taube et al. 2010)) with exception to the murine norovirus in which a fourth ORF was identified (Thackray et al. 2007).

In RHDV, ORF1 comprises nucleotide residues 10 to 7044 while ORF2 is 351 nucleotides-long, from nucleotide residues 7025 to 7378, slightly overlapping ORF1. Together these two ORF2 cover nearly 99% of the genome (Meyers et al. 1991), which also encodes the 5' and

3' untranslated regions (5'-UTR and 3'-UTR).

ORF1 encodes a polyprotein of 257 kDa (Meyers et al. 1991) that is cleaved into several non-structural proteins (such as helicase, RNA-dependent RNA polymerase and protease) and a VP60-like protein (Meyers et al. 1991; Parra et al. 1993), lacking the two aminoterminal residues with regards to the 60 kDa protein translated from the sgRNA (Parra et al. 1993) that constitutes the major viral antigen.

The ORF2 encodes the VP10, a putative minor structural protein of 117 aa (Sibilia et al. 1995).

The 257 kDa polyprotein comprises the non-structural and major capsid proteins in the order NH_2-NS1-NS2-NS3-NS4-NS5-NS6-NS7-VP60-COOH, also designated as NH_2-p16-p23-p37(helicase)-p29-p13(Vpg)-p15(cysteine protease)-p58(RdRp polymerase)-VP60-COOH (Meyers et al. 1991; Boga et al. 1992; Martín Alonso et al. 1996; König et al. 1998). The p60 is cleaved into p23 and p37. The p41 protein gives rise to products of 23 (p23/2) and 18 kDa (p18) or to polypeptides of 29 kDa (p29) and 13 kDa (p13/p14, also referred as VPg) (Meyers et al. 2000). The p72 represents a bifunctional fusion protein composed of the viral cysteine protease (p15) and the RNA-dependent polymerase (RdRP, p58) (Wirblich et al. 1996; Meyers et al. 2000)

The subgenomic RNA (sgRNA also) encodes VP60 being responsible for the synthesis of most of VP60 assembled into mature virions (Parra et al. 1993; Sibilia et al. 1995), and the minor structural protein VP10.

Therefore, major capsid protein synthesis may follow two distinct pathways, one through the processing of the polyprotein precursor translated from the gRNA producing a VP60-like protein of unknown function, and the second by translation of the sgRNA (Meyers et al. 1991; Parra et al. 1993).

The helicase and the RNA-dependent RNA-polymerase - RdRp (which catalyzes VPg uridylation) are involved in the replication of the viral RNA. The protease is involved in the proteolytic processing of the polyprotein. The function of the non-structural proteins p16, p23 and p23 is still unknown.

The gRNA express products during the intermediate and late stages of infections such as structural or movement proteins (Miller and Koev 2000). The sgRNA is synthesized during the viral replication so the RdRp is translated directly from the genomic RNA of positive-strand RNA (Miller and Koev 2000). The sgRNA and the gRNA are polyadenylated and covalently linked at the 5'end to the Tyr-21 residue of VPg (Machín et al. 2001; Meyers et al. 1991), a 115 aa viral protein with a putative role in genome synthesis initiation (Machín et al. 2001).

Rabbit haemorrhagic disease (RHD) can be caused by one of two distinct lagoviruses,

namely the RHDV/a (hereafter referred to as *Lagovirus europaeus* GI.1 or simply GI.1) and RHDV2 (from here referred to as *Lagovirus europaeus* GI.2 or simply GI.2).

The first, named *Lagovirus europaeus* GI.1 emerged in Wuxi City, in the last quarter of 1983 in domestic rabbits imported from Germany to the Jiangsu province in China (Liu et al. 1984; Parra and Prieto 1990), being the first genotype described of rabbit hemorrhagic disease virus (RHDV) (Liu et al. 1984; Le Pendu et al. 2017), that causes a fatal rabbit disease in adults and subclinical disease in rabbits younger than 4-6 weeks (Chasey 1997; OIE 2018). In the next 12 months after its emergence in China, the virus killed over 140 million rabbits in China. The virus spread to Korea in less than one year and reached all the Europe only two years later (Xu 1991; Rouco et al. 2019). Within ten years, the virus became endemic in Europe, leading to a severe reduction of wild rabbit populations, especially on the Iberian Peninsula (Argüello et al. 1988; Villafuerte et al. 1995; Delibes-Mateos et al. 2007) but also impacting industrial cuniculture in both Europe and North Africa (Morisse et al. 1991). In 1988, the virus reached North America and Mexico, being shortly eradicated due to the absence of a susceptible wild rabbit population (Gregg et al. 1991). The first cases in wild rabbits were reported in Spain in 1988 (Arguello-Villares et al. 1988) and then in Madeira Island (1988) and the Azorean archipelago (Faial in 1988, São Jorge in 1989 and Santa Maria in 1990) (reviewed in (Duarte et al. 2014; Duarte et al. 2015)). The disease reached continental Portugal in 1989, France in 1988 and the following year widespread throughout Europe (Morisse et al. 1991; Le Gall et al. 1998). In the next years, the disease was reported also in Mexico, Russia, the Middle East, Africa, India, and North America, spreading worldwide and arriving to Cuba and Uruguary.

The second genotype referred to as RHDVb/RHDV2 and formally as *Lagovirus europaeus* GI.2, is genetically related to, but distinct from RHDV, and emerged in France in April 2010 in rabbits (Dalton et al. 2012; Le Gall-Reculé et al. 2013). RHDV2 quickly replaced the circulating strains of RHDV in most European countries, both in the wild and domestic populations (Calvete et al. 2014; Duarte et al. 2018; Duarte et al. 2021). Similarly to the *Lagovirus europaeus* GI.1, GI.2 reached almost all over the world, namely Spain (Dalton et al. 2012), Italy (Le Gall-Reculé et al. 2013), Portugal (Abrantes et al. 2013), Germany (information on the FLI, 10|21|2013), England and Wales (Westcott et al. 2014), Scotland (Baily et al. 2014), Azorean archipelago (Duarte et al. 2015), Australia (Hall et al. 2015), Finland, Tenerife Island (Martin-Alonso et al. 2016), Tunisia, the Scandinavian countries of Norway, and Sweden (Neimanis et al. 2018), the Ivory Coast, Canada. By the end of 2016, the virus was also detected in the Madeira archipelago (Carvalho et al. 2017) and more recently in China (Hu et al. 2021) and in several states of the USA (Asin et al. 2021).

However, in the last decade, several cases of RHDV2 disease have been reported in the

European hare (*Lepus europaeus*) in France (2013) (Puggioni et al. 2013), Spain (2014), Italy (2012) (Velarde et al. 2017), United Kingdom (2018 and 2019) (Forrester et al. 2009), Australia (2015) (Hall et al. 2017) Sweden (2016 and 2017) and the Netherlands. RHDV2 was also reported in the Cape hare (*Lepus capensis*) on the island of Sardinia (2011), and in the Italian hare (*Lepus corsicanus*), in Sicily (Camarda et al. 2014). Recently, the RHDV2 was found in one Iberian hare (Velarde et al. 2021).

Some evidences of disease's resistance due to genetic co-evolution of rabbits and myxoma virus (Alves et al. 2019) or due to crossreaction with other non-pathogenic calicivirus antibodies are being reported (Elsworth et al. 2012) being the cross-protection conferred by the infection with non-pathogenic Australian rabbit calicivirus one factor also involved in the reduction of RHD mortality in Australia and other countries where the non-pathogenic calicivirus are reported (Strive et al. 2013; Elsworth et al. 2014).

Since 2017 (Le Pendu et al. 2017), the different lagovirus were divided into two main gene groups: related to RHDV (GI) or related to European brown hare syndrome virus (EBHSV, GII). Based on the genetic variability, namely in the major capsid protein gene - VP60/VP1 - RHDV GI genogroup can be divided into 4 genotypes: GI.1, GI.2, GI.3 and GI.4. Based on phylogenetic and genetic distances, the GI.1. genogroup can also be divided into different antigenic variants (GI.1 a-d) (Le Pendu et al. 2017). According to with this nomenclature, the GI.3 genotype is represented by RCV-E1 (*Lagovirus europaeus*/GI.3), and genotype GI.4 is represented by RCV-A1 (*Lagovirus europaeus*/GI.4) and RCV-E2 (*Lagovirus europaeus*/GI.4d), being this two groups considered, *sensu latu*, as non-pathogenic rabbit calicivirus-like viruses (RCV-like viruses).

The GII.1 genogroup contains two genotypes, namely the GII.1 containing the EBHSV and the GII.2. to which belongs the non-pathogenic strains of lagoviruses infecting hares, called HaCV. Other variants were described such as GII.1a (G1/group A), GII.1b (GI.3/group B), and GII.1c (G2/group B) (Lopes, Capucci, et al. 2014; Le Pendu et al. 2017).

The genotype GI.1 causes a highly lethal disease (70-90% of mortality and 80-90% of lethality), with an incubation period of 1-3 days, affecting animals older than 6 weeks, significantly reported only in domestic and wild rabbits (Lavazza et al. 1996). With this genotype, around 5-10% of the rabbits show a subacute-chronic clinical course (OIE 2018).

The pathogenic RHDV/RHDVa may have evolved from non-pathogenic or less pathogenic strains circulating in European rabbits before the first outbreak of RHD in China (Capucci et al. 1996; Moss et al. 2002; Forrester et al. 2007; Abrantes et al. 2020), probably deriving since 200 years ago from the same ancestor of Rabbit calicivirus-like viruses (Kerr et al. 2009). A second hypothesis for the emergence is the spillover from caliciviruses found in micromammals sympatric to the European rabbit (Merchán et al. 2011; Le Gall-Reculé et al.

2013; Esteves et al. 2015). Besides the similar clinical pattern with the EBHSV detected for the first time in 1980 in European hare, the similarity of VP1/VP60 is about 76% indicating that the RHDV did not evolve from this virus (Wirblich et al. 1994; Nowotny and Bascun 1997). In 2010, a new genotype of RHDV was identified in France (Le Gall-Recule et al. 2011), referred to as RHDV2 or RHDVb (Dalton et al. 2012; Le Gall-Reculé et al. 2013) and nowadays known as *Lagovirus europaeus* GI.2 or simply GI.2 (Le Pendu et al. 2017).

This new genotype presents a phylogenetically different genotype with more than 15% of divergence from other lagoviruses, including RHDV and the non-pathogenic RCVs and HCVs, besides a similar genome organization (Dalton et al., 2015).

GI.2 differs also from GI.1 in antigenic profile, the apparent lower mortality (5-70%, 20% in average) (Le Gall-Recule et al. 2011; Le Gall-Reculé et al. 2013; OIE 2018), because affects kittens of just 11 days (Dalton et al. 2012), course of disease of 3-5 days and a higher proportion of rabbits showing subacute-chronic disease comparing with the previous genotypes (OIE 2018). The GI.2 has a broader host range, being detected in the *Lepus europaeus* (Velarde et al. 2017), *L. capensis* (Camarda et al. 2014), *L. timidus* (Aleksija S Neimanis et al. 2018) and *Lepus granatensis* (Velarde et al. 2021), Lepus californicus and *Sylvilagus audubonii* (Asin et al. 2021), in small mammals species (Calvete, Mendoza, M.. Sarto, et al. 2019) and Eurasian badgers (Abade dos Santos et al., 2021). The GI.2 genotype replaced the genotype GI.1 in the Iberian Peninsula and other regions (Calvete et al. 2014; Dalton et al. 2014; Lopes et al. 2014; Mahar et al. 2018), showing a high recombination rate with other pathogenic and non-pathogenic RHDV strains (Silvério et al. 2018) and giving rise as well to very highly pathogenic strains with mortalities close to 100% in adults and kit rabbits (Calvete et al. 2021).

1.5.2. Pathophysiology

The suggested primary sites of RHDV replication are the epithelial cells of the upper respiratory and digestive tracts, also the most probable entry doors, while hepatocytes are the major site of RHDV replication (Ruvoën-Clouet et al. 2000; Guillon et al. 2009). The viral genome is released into the cell cytoplasm leading to the direct translation of the viral proteins. The lack of m7G cap structures linked to the mRNA (present in most viruses) in the caliciviruses, may indicate that the viral genome linked protein VPg play a crucial role in the translation initiation (Daughenbaugh et al. 2003; Meyers and Tu 2003). The VPg acts as a cap substitute or analogue, interacting with translation initiation factors eIF4E and/or eIF3 (Daughenbaugh et al. 2003; Goodfellow et al. 2005).

While the translation of ORF1 encoding polyprotein precursor occurs at the initiation codon

AUG, the translation of ORF2 encoding VP10 starts by an unusual mechanism of reinitiation after the termination of translation of the preceding major capsid protein VP60 (Meyers and Tu 2003), depending ot the last 84 nucleotides of ORF1. VP10 can induce hepatocyte apoptosis and virion release and dissemination (Liu et al. 2008).

RHDV binds to ABH histo-blood group antigens (HBGAs) on epithelial cells of the upper respiratory and digestive tracts (Ruvoën-Clouet et al. 2000; Family et al. 2001), the HBGAs act as attachment factors or ligands for RHDV, rather than the main cellular receptor (Nyström et al. 2011).

The incubation period of RHD induced by GI.1. ranges from 1 and 3 days (Arguello-Villares et al. 1988; Marcato et al. 1991) and by GI.2. ranges between 3 and 9 days with death occurring 12–36 hours after the onset of fever. However, there are reports of subacute and chronic infections caused by GI.2. (Le Gall-Reculé et al. 2013).

Mortality associated with RHDV2 infection varies in different reports and depends on the strains. It was initially considered lower than classic RHDV, but more virulent GI.2 strains become known with mortality, incubation period and disease course similar to GI.1 (Capucci et al. 2017). This genotype is currently reported in more than 40 countries in Europe, Oceania, Africa, North America and Asia (Rouco et al. 2019; Abade dos Santos et al. 2021; Ambagala et al. 2021; Duarte et al. 2021; Fukui et al. 2021). The virus is endemic in most parts of the world where European rabbits live naturally or are domesticated (OIE 2018).

In North America, the virus is very recent in California and many states of USA affecting several species of rabbits and hares and many native predators such as the mountain lions (*Puma concolor*) and bobcats (*Lynx rufus*), coyotes (*Canis latrans*), and golden eagles (*Aquila chrysaetos*) (CDFW 2021). GI.2. constitutes a potential threat to the conservation of five lagomorph species whose conservation status rises concerns, including the endangered Riparian brush rabbit (*Sylvilagus bachmani riparius*) (CNDDB 2021).

In GI.2. infection the clinical signs almost overlap those induced by different RHDV genotypes, including fever (>40°C) with hypothermia and convulsions before death, anorexia, tachypnoea, epistaxis, lethargy, epistaxis, or sudden death (Abrantes et al. 2012). The infection culminates in multi-organ failure resulting from the development of DIC syndrome (Alonso et al. 1998; Trzeciak-Ryczek et al. 2015). At necropsy and histopathology, liver swelling, congestion and/or discolouration (pale yellow, greyish or dark reddish discoloured), cloudiness and fragility, and pulmonary oedema are observed. Kidneys appear congested and enlarged with sub-capsular lesions including ecchymosis and haemorrhages, splenomegaly, with the main microscopic lesion being severe acute hepatitis with hepatic necrosis, mainly peripheral and intra-glomerular capillary hyalin thrombi. Haemorrhages and congestions can be seen in several organs, particularly in the lungs, heart, and kidneys, mild

to moderate inflammatory infiltrate consists of lymphocytes in portal spaces and sinusoids, and granulocytes in sinusoids, tracheal and pulmonary lesions are mainly of the hyperaemic-oedematous type with congestion of the tracheal sub-mucosal capillaries and leukocyte infiltration (Arguello-Villares et al. 1988; Argüello-Villares 1991; Marcato et al. 1991; Ueda et al. 1992; Abade dos Santos 2018; Fukui et al. 2021).

Death may occur rapidly within 12 to 48 hours after exposure (Arguello-Villares et al. 1988; Capucci et al. 1991; Marcato et al. 1991; McIntosh et al. 2007). In subclinical-chronic disease, severe and generalised jaundice, cachexia and lethargy may be observed, with death occurring after 1-2 weeks of the symptom onset or surviving after seroconversion. The IgM antibodies appear within 3 days, and 2-3 days later, IgA and IgG can be detected (Barbieri et al. 1997). In animals that survive the infection, the viral RNA can be detected by RT-PCR up to 15 weeks after the infection in blood and faeces (Gall et al. 2007).

The physiopathology of the disease can be resumed in two phases (Müller et al. 2021):

i) Disturbed physiology due to extremely rapid RHDV replication in the liver, spleen and kidney within 1.5 to 3 days after infection, reaching up to 108 copies of viral RNA per g of liver tissue (Gall et al. 2007). The disease progresses with increased apoptosis of hepatocytes and liver endothelial cells (Alonso et al. 1998). The coagulation is disturbed and micro-thrombi and disseminated intravascular coagulation occur due to an increase in liver enzyme activity (such as creatinine kinase), prothrombin time, fibrin degradation and decreased antioxidant enzymes and antithrombin III activity (Ueda et al. 1992). Respiratory acidosis and hypoglycemia occur. All these alterations result in multi-organ dysfunction and death (Sanchez-Campos et al. 2004).

ii) Disturbed immune response, mainly due to systemic apoptosis of lymphocytes, especially T-cells within 48-72h after infection. The apoptosis of T- and B-cells was reported in the liver and blood, along with the decrease of regulatory T-cells and infiltration of neutrophils of the infected organs (Marques et al. 2010; Niedźwiedzka-rystwej and Deptuła 2012; Teixeira et al. 2012; Niedźwiedzka-Rystwej et al. 2013). Due to the pathogenicity of GI.2 in very young rabbits and given the immature immune system at this stage, especially of the B-lymphocyte ontogeny and maturation (Knight and Winsteadt 1997), the innate immune takes on great importance limiting the initial replication of the virus. In the non-hemagglutinogenic and hemagglutinogenic strains, the apoptosis of granulocytes and peripheral blood lymphocytes is initiated from 4-8 p.i. and 12h p.i., respectively, and intensified in both cases up to 24-36h p.i. (Niedźwiedzka-rystwej and Deptuła 2012).

During the GI.1. infection, an increase of TNF-α, IFN-α, IFN-γ, TGF-β, and interleukin-1, IL-

6, IL-8, and IL-10 occurs (Teixeira et al. 2012; Semerjyan et al. 2019). In the liver, a decrease in hepatocyte growth factor (HGF) expression, and an increase in TNF-α, TGF-β, IL-1β and IL -6 were measured (Sanchez-Campos et al. 2004; García-Lastra et al. 2010; Tunon et al. 2011). In the peripheral blood lymphocytes, a decrease in IL-1β, IL -2, and IL -18 activity, and an increase in IL-6, IL -8, IL-10, TNF-α, and TGF-β (Trzeciak-Ryczek and Tokarz-Deptu 2016; Trzeciak-Ryczek and Tokarz-Deptu 2017) was observed.

In particular, after GI.2. infection, genes of the major histocompatibility class I complex, as well as interferon-induced genes, were downregulated which correlates with the limited resistance of juveniles. In the case of adults, the MHC class II genes were upregulated, related to an induction of Th2 immune response, still insufficient to prevent the RHD due to rapid evolution (Neave et al. 2018).

The virus replicates first in the peripheral epidermal cells and then in cells deeper in the dermis (Spiesschaert et al. 2011). The antigen is recognized in the primary infection site by pathogen recognition receptors (PRRs) on the surface or in the endosomes of dendritic cells or other myeloid cells (Bousarghin et al. 2005; Ulsenheimer et al. 2005; Mohsen et al. 2017; Zepeda-Cervantes et al. 2020). The virus is internalized, activating and maturing the dendritic cells. These mature cells process and present virus-derived peptides into the major histocompatibility complex (MHC) class I or class II molecules to the T-cell receptor complex of CD8+ or CD4+ T-cells, respectively. The virus enters subsequent in circulation via the lymphatic and vascular systems, spreading into numerous secondary organs. This interaction results in activation and proliferation followed by adaptive effector function via Th1 and/or Th2 responses (Müller et al. 2021). The replication of cytotoxic CD8+ T-cells (Th1 response) which lyse virus-infected cells or abrogate the viral replication through IFNγ-promoted Type I IFN responses limits the viral replication. On the other side, the Th2 response leads to producing of neutralizing antibodies blocking the viral epitopes (Bachmann and Jennings 2010; Serradell et al. 2019). The Caliciviruses also present mechanisms, namely viral proteins able to block the intracellular processing and presentation of antigen-presenting cells (Peñaflor-téllez et al. 2019).

The RHDV is transmitted by direct contact (via oral, nasal or conjunctival mucosae), exposure to infected carcass, hair, food or water and through human activities and mechanical vectors (e.g. flies and other insects), the last ones often the source of long-distance spread (Chasey 1997; Fenner 2010; OIE 2018). Very low doses are sufficient to infect a naïve host (Gehrmann and Kretzschmar 1991). Some studies analyzed the spread rate of GI.1, estimating it between 180-4970 km/year, is lower in Spain than in Australia (Villafuerte et al. 1995; Kovaliski 1998), not existing statistically significant difference in the spreads of the two genotypes, although GI.2 was slightly faster (Aguayo-adán et al. 2021).

1.5.3. Prophylaxis

As a naked virus, the different RHDV genogroups, as happens with the Calicivirus in general, have a high resistance to environmental conditions (Henning et al. 2005). The virus can resist for 10–44 days kept dried on cotton tape, while injected into the bovine liver (mimetizing the virus resistance in organic material) keep viable after 3 months. At 4ºC in an organic suspension, the resistance was of 225 days (Šmíd et al. 1991) or around 413 days at -5ºC (Xu 1991).

Liver samples collected from rabbit carcasses at 22º up to 20 days post mortem were able to infect and kill susceptible rabbits, while after 26 days did not result in mortality (McColl et al. 2002). The resistance is such higher that the virus can resist to pH=3.0 for 60 minutes as well as 1h at 37ºC or 12h at 4ºC in 0.4% formaldehyde (Smid et al. 1989; Xu and Chen 1989).

Several vaccines were developed against RHDV and more recently for RHDV2. Initially, the vaccines of first-generation (e.g. Arvilap, Laboratorios Ovejero; FILAVAC VHD K C+V, Filavie S.A.S; ERAVAC, Hipra) consisted of inactivated virus from a clarified liver suspension of experimentally infected rabbits, completed with an adjuvant. The active or passive immunity acquired by infection or vaccination using a GI.1 strain does not confer appropriate immunity against GI.2 (OIE 2018). The first injection is generally given at 2-3 months with a yearly booster. For the majority of vaccines, the immunity starts after 7-10 days. Rabbits have a haemochorial placenta, through which antibodies pass and in part in the lactation period (Furukawa et al. 2014).

Currently, second-generation vaccines are available, namely a recombinant myxoma virus expressing VP60 of both RHDV and RHDV2. Many experimental studies were performed without a final commercial product, using RHDV capsid protein expressed in *Escherichia coli* (Boga et al. 1994; Guo et al. 2016), adenovirus (Fernández et al. 2011), vaccinia virus (Bertagnoli et al. 1996), baculovirus (Nagesha et al. 1995; Dalton et al. 2021), yeast (Boga et al. 1997; Fernández et al. 2013), and even in plants (Bertagnoli et al. 1996; Viaplana et al. 1997; Marti and Biologi 1999). However, for example, the protein expressed in *E. coli* is highly insoluble and of low immunogenicity (OIE 2018). The VP60 expressed in the baculovirus/Sf9 cell expression system, self-assembles into VLPs structurally and antigenically identical to RHDV, able to induce immunity using the simple VLPs, or by using recombinant vaccinia and canarypox, administered either intramuscularly or orally (OIE 2018).

1.5.4. Diagnosis

The recommended techniques for RHD diagnosis are detailed in the OIE Terrestrial Manual of 2018 (OIE 2018), and they will only be briefly referred to here. Direct methods include the observation of viral particles or the detection of viral antigen, protein or RNA. The immune response to infection can be investigated by the detection of antibodies (indirect method of diagnosis).

To date, there is no known method of propagation of RHDV *in vitro*, either using primary cell cultures or established lines (OIE 2018). Diagnosis is mainly made by necropsy, histopathology and molecular methods, taking into account that the necropsy examination is neither sensitive nor specific, often not observing lesions, and that there are no pathognomonic lesions (Abade dos Santos, 2018).

In general, the liver contains the highest viral titre (from 10^3 LD50 [50% lethal dose] to $10^{6.5}$ LD50/ml of 10% homogenate) being the organ of choice for viral detection in both RHDV and EBHSV. The viral loads are directly proportional to vascularization, being spleen and serum alternative diagnostic materials, particularly in subacute/chronic disease (OIE 2018).

The initial preparation of the material consists of the mechanical homogenization in phosphate-buffered saline solution (PBS, pH 7.2) at 5-20% w/v and clarified by centrifugation at 3-8,000 rpm for 5-15 min (OIE 2018). At this stage, the supernatant can be directly submitted to the HA test or enzyme-linked immunosorbent assay (ELISA). For electron microscopy is advisable intermediary centrifugation at 10-12,000 rpm for 5 to 15 min and then the ultracentrifugation. For PCR, viral RNA may be directly extracted from tissues samples, with all the steps, preferable carried out at 4°C (OIE 2018).

During a subacute or chronic course of the disease, the antibody response triggers the viral clearance in the liver and spleen. In these cases, an RHD virus-like particle (VLP) is present, resulting from the auto-assembly property of the capsid pentamers (Laurent et al. 1994), instead of RHDV in the spleen and liver, lacking the outer shell on the viral capsid made up by the half C-terminal portion of the VP60 (Capucci et al. 1991; Granzow et al. 1996; Barbieri et al. 1997). For this reason, these samples are negative in the haemagglutination test and undetected by anti-RHDV monoclonal antibodies directed to outer conformational epitopes (Capucci et al. 1995).

During RHD serology analyses several non-pathogenic RHDV-related lagoviruses (rabbit calicivirus – RCV) have been isolated and partially characterised in Europe and Oceania (OIE 2019). These "enteric viruses" induce a serological response that may interfere with and complicate RHD serological diagnosis (Nagesha et al. 2000; Robinson et al. 2002).

1.5.4.1. Direct methods

Electron microscopy

Negative staining uses heavy metals salts to provide contrast to virions, which appear translucent (Schramlová et al. 2010) and stand out from the background, providing morphological information on symmetry and capsomer arrangement. Negative-staining electron microscopy can be performed using the so-called 'drop method', but it presents lower sensitivity, being ideal to ultracentrifuge the sample to concentrate the viral particles (OIE 2018). Using this technique, naked particles become visible, 32-44 nm in diameter, inner shell 25-27 nm in diameter, delineated by a rim from which ten short peripheral projections radiate, regularly distributed. The VP60 includes the N-terminal arm (NTA), the shell (S), and the protrusion (P) forms in this particle's characteristic cup-shaped depressions. Other particles, named smooth particles (s-RHDV) lost the external portions, appearing perfectly hexagonal and smaller, with only the capsid rim visible (Capucci et al. 1991; Granzow et al. 1996; Barbieri et al. 1997). A more sensitive method is the immuno-EM technique (IEM) (Lavazza et al. 2015) applying serum from a convalescent animal or monoclonal antibodies with the Immunogold technology. The IEM proved to be of higher sentivity and specificity than haemagglutination (HA) and nearly equivalent to enzyme-linked immunosorbent assay (ELISA) (Capucci et al. 1991).

Cell and Tissues Immunolabelling

Immunocytochemistry/Immunohistochemistry/Immunofluorescence techniques can be performed in fresh cells, formalin fixed tissues and cryo-sections, and allows the characterization of virus distribution in the different cells and tissues, using various detection systems including the Avidin-Biotin Complex (ABC)-Peroxidase method. In the liver, for example, it is observed an intense nuclear staining and diffuse cytoplasmic staining of hepatocytes, mainly in the periportal areas, and also positive staining of macrophages and Kupffer's cells in the liver, macrophages in the lungs, spleen, lymph nodes and other organs and the glomerular mesangial cells in the kidney (Stoercklé-Berger et al. 1992). Ideally, monoclonal antibodies should be used or hyperimmune sera produced in a species other than the species' sample to be diagnosed.

In-vitro and in-vivo virus isolation

The RHDV did not propagate in any cell line. Rabbit inoculation remains the only way of isolating, multiplying and titrating the infectivity of RHDV, in the absence of a cell culture platform. This technique is not currently used for diagnosis purposes.

Antigen detection

The Hemagglutination test was the first used for routine laboratory diagnosis of RHD (Liu et al. 1984). The virus haemagglutinate many species of red blood cells, bein the human Group O red blood cells the ideal choice (OIE 2018). Haemagglutination (HA) test is a simple diagnosis and titration test that can be used, bearing in mind that some HA-negative RHDV variants exist (OIE 2018). The RHDV2 showed an HA activity similar to RHDV/RHDVa (Le Gall-Reculé et al. 2013). In general, agglutination at an end-point dilution of >1/160 is considered to be positive. When the titre is lower, the sample should be tested using other methods. Compared with ELISA or EM, the HA presents a sensitivity 10% lower (OIE 2018). False negatives occur mostly in subacute/chronic forms of the disease and whenever virus particles have undergone proteolytic degradation (OIE 2018). Taking into account that EBHS in hares is often subacute or chronic and therefore the virus has the antigenic and structural characteristics typical of the VLPs (Capucci et al. 1991), the technique, even modified according to the OIE Terrestrial manual, just detects 50% of samples (OIE 2018), as the sample can be used a 10% (w/v) tissue homogenate of liver, spleen, lungs or kidneys (OIE 2018). The haemagglutination can be positive as early as 2h post-infection and rise rapidly with a maximum titre at death ($10x2^{14}$ to $10x2^{16}$) (Yang 1989; Yang et al. 1989), considering as positive a end-point dilution greater than 1/160 (Ahmad et al. 2011).

The western blot technique can be useful for the diagnosis of doubtful samples in other techniques, particularly in the presence of s-RHDV particles. Enables the identification of specific proteins (Mahmood and Yang 2012). This technique can detect the RHDV 6S subunits (Capucci et al. 1995) and the denatured VP60 structural major protein or its proteolytic fragments, sizing between 50 to 28 kDa (OIE 2018). The single structural protein of RHDV – VP60 - sizes 60 kDa and fragments of the VP60 associated with the transition from RHDV to s-RHDV sizes between 28-41 kDa.

As starting material, homogenates prepared as described above can be used with a further concentration by ultracentrifugation through a 20% (w/w) sucrose cushion, being used as both the supernatant and the pellet (Capucci et al., 1995). In general, the proteins are denatured by heat in the presence of sodium dodecyl sulphate and betamercaptoethanol,

separated by a polyacrylamide gel electrophoresis (SDS/PAGE), and transferred by electroblotting onto nitrocellulose or polyvinylidene flouride (PVDF) membranes (OIE 2018). The proteins are detected using polyclonal antibodies or Mabs that recognize continuous epitopes. The membranes are then washed and incubated with anti-species alkaline phosphatase-labelled immunoglobulins. Finally, chromogenic substrate (5- bromo-4-chloro-3-indolylphosphate nitro blue tetrazolium) is added (OIE 2018). Different adaptations of this protocol can be made including using fluorescent secondary antibodies.

Other technologies such as immunochromatography were also developed to the diagnosis of RHDV and RHDV2 (Dalton et al. 2017; Fresco-Taboada et al. 2022).

Molecular methods – DNA detection

The PCR, coupled to reverse transcription (RT)-PCR, is currently the ideal rapid diagnostic test for RHD (Guittre et al. 1995; Gould et al. 1997). The first described RT-PCR, using a conventional system was described in 1995 (Guittre et al. 1995), amplifying an N-terminal portion of RHDV vp60 and being 10^4 more sensitives than ELISA.

At this moment, several real-time methods using a one-step strategy were described, using primers and probes able to amplify all RHDV genogroups and RHDV2 or specific to RHDV2 (OIE 2019) including a real-time method (Duarte et al. 2015), the last able to detect 9 copies of the virus in almost all the organ specimens of an acutely infected animal (Abade dos Santos, 2018). Main techniques to detect the different RHDV genotypes, including the non-pathogenic RCV, and EBHSV, by conventional or real-time PCR techniques, are reported in the OIE manual, being essentially targeted to the VP60 gene. Other techniques for RHDV genotype detection were also described before the *Lagovirus europaeus* GI.2. emergence, using loop-mediated isothermal amplification (LAMP) (Yuan et al. 2013) or SYBR green-based real-time PCR (Liu et al. 2015).

The new massive sequencing techniques allowed the identification of new viruses including some beneficial or commensal viruses. In other words, these techniques allowed the sequencing of full genomes of many viruses, more efficiently than using previous technologies, mainly Sanger sequencing.

These techniques can also have a higher sensitivity than RT-PCR and might overcome RT-PCR primer issues, in case of rapidly evolving viruses that lead to primers and probes mismatching.

The first *hybridization in situ* technique for detection of RHDV was developed in 1998 (Gelmetti et al. 1998) using two RNA probes (sense and antisense) transcribed in vitro and UTP-digoxigenin-labelled (Gelmetti et al. 1998), able to detect RHDV as early as 6-8h after

infection. This technique is mainly used for research purposes.

1.5.4.2. Indirect methods

The humoral response has the highest relevance in the response to RHD, so the determination of specific antibody titres after surviving disease or vaccination is predictive of the animal's protection (OIE 2018). After infection, B lymphocytes (determined as CD19+) increased constantly from 8 h to 52 h post-infection with a peak of IgM within 2 weeks, and a slower IgG response that persisted for months (Niedźwiedzka-Rystwej and Deptuła 2010; P. Niedźwiedzka-Rystwej et al. 2013; Müller et al. 2019). Due to antigenic differences existing between the RHDV/RHDVa and RHDV2 genotypes, specific immunological reagents can be used to differentiate the response against these two viruses.

Many techniques have been developed with the same technical fundament, namely the haemagglutination inhibition (Liu et al. 1984), indirect ELISA (available commercially), solid-phase ELISA, sandwich ELISA and competitive ELISA (Capucci et al. 1991) used by the OIE Reference laboratory. In some particular cases, a combination of ELISA techniques that distinguish IgA, IgM and IgG antibody responses can be also used (Cooke et al. 2000). It should be taken into account that the diagnosis of chronic RHD may be problematic due to the presence of high anti-RHDV antibody titres in the samples, causing possible false-negative results in ELISA and especially in HA tests.

 The protocols recommended by the OIE Reference laboratory can be consulted in the OIE Terrestrial Manual, 2018.

Commercial and laboratory ELISA have been developed, including the competitive ELISA by the OIE Reference Laboratory. The most recent ELISA methods use monoclonal antibodies and are specific to the different RHDV genotypes. ELISA has proved higher sensitivity and specificity than HA and the combined use of the monoclonal and polyclonal antibodies allows for the differentiation of RHDV from EBHSV and between different serotypes, The purified RHDV, or ideally VLPs, can be used as antigen for plate coating.

1.6. Myxoma viruses

1.6.1. Emergence and spatiotemporal distribution

Myxoma virus (MYXV), the etiological agent of myxomatosis, is a double-stranded DNA *Leporipoxvirus,* belonging to the *Chordopoxvirinae* subfamily, family *Poxviridae.* Myxomatosis is an endemic disease of South American rabbits and was first described in

laboratory rabbits in 1898, in Uruguay (Sanarelli G. 1898). The disease is characterized by the presence of nodules in the skin surrounding the eyes, nose, mouth, ears, and genitalia. Conjunctivitis accompanied by purulent discharge is frequently found as a signal of disease (Rivers 1930; Arthur and Louzis 1988). Regardless of the two disease presentations (classic, nodular or typical form of disease and respiratory or amyxomatous form), myxomatosis was considered a rabbit disease for many decades, with some scarce reports in European hares (Collins 1955; Barlow et al. 2014).

Myxoma virus (MYXV) is the type species of the *Leporipoxvirus* genus (family: *Poxviridae*; subfamily *Chordopoxvirinae*) and causes an innocuous cutaneous fibroma, which persists for some weeks in its natural host, the South American tapeti or forest rabbit (*Sylvilagus brasiliensis*), being passively transmitted on the mouthparts of biting arthropods. This is a textbook example of a perfect evolutionary climax between the pathogen and host (Burnet 1968).

Myxomatosis was described for the first time in laboratory rabbits, in 1896, by Giuseppe Sanarelli, in Montevideo, Uruguay (Fenner and Ratcliffe 1965). In contrast, infection of the European rabbit (*Oryctolagus cuniculus*), by MYXV causes a generalized, lethal disease (called myxomatosis) characterized by a swollen head, eyelids and ears, cutaneous lesions over the body, ears and legs, anogenital oedema, blepharoconjunctivitis and mucopurulent ocular and nasal discharge. This disease was first described at the Institute of Hygiene in Montevideo, Uruguay in 1896 (Sanarelli G. 1898).

The following leporipoxviruses (Kerr et al. 2015) and respective natural hosts are presently known:

a) Myxoma virus – Tapeti (*Sylvilagus brasiliensis*)

b) Californian myxoma virus – Brush rabbit (*Sylvilagus bachmani)*

c) Rabbit (Shope) fibroma virus - Eastern cottontail (*Sylvilagus floridanus*)

d) Squirrel fibroma vírus - Eastern gray squirrel (*Sciurus carolinensis*)

e) Hare fibroma virus - European brown hare (*Lepus europaeus*)

f) Western squirrel fibroma virus - Western gray squirrel (*Sciurus griseus griseus*)

The Lausanne (Lu) strain of MYXV (Brazil/Campinas 1949), considered *de facto* as the international reference MYXV strain (ATCC code VR-115), has a double-stranded DNA (dsDNA) genome with 161,777 bp of size, with closed single strand hairpin termini. The genome encodes 158 unique open reading frames (ORFs), 12 duplicates in the 11,577 bp terminal inverted repeats (TIRs) (Cameron et al. 1999; Morales et al. 2009).

The viral genome is encapsidated in a brick-shaped virion, and the replication cycle occurs in the cytoplasm of infected cells where a spectrum of host-interactive immunomodulatory

proteins are expressed (Kerr and McFadden 2002).

Genes encoding proteins involved in replication and structure are relatively conserved among other poxviruses and tend to be located in the central part of the genome, while genes at the termini tend to encode host-range and virulence factors (Cameron et al. 1999; Upton et al. 2003). The function of 42 genes are related to host range or immunomodulation (Kerr 2012). MYXV infects only rabbits and European Brown Hares (*Lepus europaeus*), the last one only occasionally with clinical signs, and is nonpathogenic in any tested hosts apart from lagomorphs. MYXV replicates in cultured cells from many species, including most human cancer cells (Fenner and Ratcliffe 1965)

The potential of myxomatosis as potential biological control of rabbits was suggested for the first time in 1918 (Aragão 1927; Fenner and Ratcliffe 1965), starting the species-specificity trials and the respective lethality in the 1930s (Martin 1936; Bull and Dickinson 1937). The virus (a strain referred to as Standard laboratory strain (SLS), grade 1 of virulence) was released in Australia, as an alternative to the release of pathogenic *Pasteurella* suggested by Luis Pasteur (Fenner and Ratcliffe 1965), but failed to show potential as biological control weapon due to limited dissemination probably due to the geographical location chosen for the viral release, a very dry country, where mosquitoes were scarce (Bull & Mules, 1944; Fenner & Fantini, 1999; Fenner & Ratcliffe, 1965). Additional introductions were made in Tasmania and Western Australia (Calaby et al., 1960; Fenner & Ratcliffe, 1965). After these first releases, the Australian rabbit population underwent losses of 99%–100%, with 400 million rabbits succumbing in just one year (Burnet, 1952; Ratcliffe et al., 1952). In the next years, the rabbit populations recovered a little, but in the 1990s, the populations were estimated at 5% and 25% of their previous level, respectively, in agricultural zones and rangelands (Williams et al. 1995). The co-evolution selection pressure reduced the efficacy of myxomatosis as a biological control in the wild rabbits. This phenomenon occurred in Australia, Great Britain and France (Bartrip, 2008; Best & Kerr, 2000).

Another strain, named Lausanne strain (Lu, also referred to as Brazil/Campinas 1949) was introduced illegally by a farmer in June 1952 in the state of Maillebois in northwestern France. The rabbits in Europe, were completely naïve to MYXV, as in Australia, and the virus successfully spread into the wild rabbit of Western Europe, Ireland and the United Kingdom (Rogers et al. 1994) including some deliberated dissemination into Britain in 1953 (Thompson and King 1994). Due to wild rabbit spread, this species' populations fell by 99% and 90-95%, respectively, in UK and France (Lloyd 1970; Rogers et al. 1994).

Since the 1980s, in Australia, the predominant viruses detected in the field are from grade 3, being the grades 1, 2 or 5, found in less than 5% of the cases (Fenner, 1983; Kerr et al., 2010; Saint et al., 2001). Since the1990s, myxomatosis is chronic enzootic in rabbit

populations in America, Southern Europe, New Zealand and Australia (Fenner & Fantini, 1999).

1.6.2. Pathophysiology

The replication of MYXV inoculated intradermally in European rabbit initiates in MHC-II+ cells at the dermal/epidermal interface. Then, the virus spreads to the draining lymph node, first replicating in cells of the subcapsular sinus and later in lymphocytes of T cell zones. Lymphocytes, and possibly monocytes, disseminate the virus to distal tissues, with low viral load detectable in the bloodstream (Fenner and Woodroofe 1953; Best et al. 2000; Best and Kerr 2000). Simultaneously, in the original inoculation site, the virus replicates in epidermal cells inducing hyperplasia and hypertrophy, disrupting the dermis and causing oedema and infiltration of mucoid material in the sub-dermis matrix – originating the myxoid tissue that is responsible for the virus's name.

The higher virus titres are found in lymphoid tissues (> 10^8 pfu/g) with lymphoid depletion in the lymph nodes and, in some strains, in the spleen. High loads are also found in secondary cutaneous lesions, the swollen eyelids and particularly in the very swollen tissues at the base of the ears, which are probably important for insect transmission. The viral loads in the lungs or liver are generally lower (Best & Kerr, 2000; Fenner & Woodroofe, 1953; Hurst, 1937).

This ability to replicate and disseminate via lymphocytes, establishing infection at distal sites, is critical to the virulence of the European rabbit (Spiesschaert et al. 2011; Kerr 2012).

The virus shedding occurs by conjunctival and nasal secretions and by eroded cutaneous lesions. Secondary bacterial infections of the upper respiratory tract and conjunctiva with gram negative bacteria (e.g. *Pasteurella multocida* and *Bordetella bronchiseptica*) are common, with infection of internal organs less probable (Hobbs 1928). When the duration of the disease is longer (i.e. infection by less pathogenic strains), pneumonia is a common outcome (Duclos et al. 1983; Marlier et al. 2000; Kerr et al. 2015).

The pathophysiological process of death by myxomatosis is still poorly understood. The key viral proteins involved in the replication and dissemination in *Sylvilagus* species cause local immune suppression in the skin, allowing the virus to persist at this level. In the European rabbit, these proteins cause profound immunosuppression. The virulence of field strains was analyzed by infecting small groups of laboratory rabbits, classifying them into five broad grades based on the case fatality rates (CFR), average survival time (AST) and clinical signs (Fenner and Marshall 1957; Fenner and Ratcliffe 1965; Fenner and Woodroofe 1965), namely :

Grade 1: CFR of 99.5%, AST ≤ 13 days

Grade 2: CFR of 95-99%, AST 14-16 days

Grade 3A: CFR of 90-95%, AST 17-22 days

Grade 3B: CFR of 70-90%, AST 23-29 days

Grade 4: CFR of 50-70%, AST 29-50 days

Grade 5: CFR less than 50%, AST not determined

1.6.3. Prophylaxis

Virus transmission occurs by direct contact, by mosquitoes or by biting arthropods mechanical transmission. Throughout these processes, the virus excreted by the eroded cutaneous lesions, conjunctivae and nasal passages can be transmitted into the upper respiratory tract or conjunctiva of other rabbits. Grade 4 strains appear to be the most transmissible (Fenner et al. 1956) since the virus remains at high titres in the epidermis for long periods. On the contrary, both grade 1 and grade 5 viruses are poorly transmitted due to the quick death of the infected animals or to the effective immune response, respectively (Fenner et al. 1956). In general, grade 4 strains predominate in the populations with no genetic resistance, and grade 3B in more resistant populations (Anderson and May 1982; Dwyer et al. 1990).

The *Spilopsyllus cuniculi,* present across Europe and other flea species such as the Spanish rabbit flea (*Xenopsylla cunicularis*), are efficient vectors of the disease. These fleas were not originally present in Australia, but their introduction increased the dissemination of the virus (Sobey and Menzies 1969; Cooke 1983). The transmission by fleas can occur throughout the year, depending on the edafoclimate conditions, whereas flying vectors such as mosquitoes, culicoides, midgs, simuliids and others are more seasonal (spring and summer).

Contrarily to RHDV, the myxoma virus can grow in cell lines and live virus vaccines have been extensively used in Europe (Arthur and Louzis 1988) due to the inefficacy of inactivated vaccines (Joubert and Leftheriotis 1972; Brun et al. 1981; Guitton et al. 2008; Ferreira et al. 2009; Marlier 2010) being a heterologous rabbit fibroma virus the first available (Fenner and Woodroof 1954). Around 10 years later, a homologous vaccine was produced using a variant named MYXV MSD strain or others like French SG33, attenuated by a passage in cell culture (Saito et al. 1964). Currently, as referred to in Section 1.5 (The Lagoviruses), the myxoma virus is also used as a vector for RHDV vaccines.

1.6.4. Diagnosis

The typical myxomatosis shows very specific lesions, particularly nodular thickening of the

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eyelids, the presence of myxomas and anogenital oedema. However, due to the existence of different degrees of virulence and different viral strains, laboratory diagnosis is important, particularly in the surveillance of the genetic evolution of the virus. Clinical diagnosis is still difficult in cases of the amyxomatous form, due to lesser ectodermal tropism (OIE 2020).

The identification of the infection must take into account that the myxoma virus has a relatively high evolutionary rate for a dsDNA virus, with an average nucleotide substitution of 1×10^{-5} per site per year that accumulates with time, in a remarkably clock-like manner. However, much of the genome is highly conserved, particularly the early, intermediate and late promoter sequences (Willer et al. 1999; Kerr et al. 2012; Kerr et al. 2013)

Commonly mutated genes are the m014L, m083L, m014L or m009L genes (Kerr et al. 2015). The disruption of m009L gene by an indel (insertion-deletion mutations) formation is observed in all but one of the recent viruses. This, and m083L disruption occur in all strains of the long branch of the phylogenetic tree that separates the 1950s from the 1990s viruses. Some viruses present further disruptions in ORF m0009L, including premature stop codons, suggesting the possibility of a pseudogene occurring. The m009L gene belongs to a gene group (comprising m006L/R, m008 L/R and m009L), which may indicate an earlier duplication event during leporipoxvirus evolution (Kerr et al. 2015).

One of the few attenuated strains whose complete genome sequence is known is the Spanish 6918, isolated in 1995 (Morales et al. 2009). This strain exhibits a disruption by indels of m009L, m036L, m135R and m148R genes, the latter two with a demonstrated role in virulence (Barrett et al. 2007; Blanié et al. 2009). Various Spanish MYXV strains present disruptions in m009L and m036L or m002L/R and m017L genes (Dalton et al. 2010; Muller et al. 2010). In some Portuguese strains, m009L is also disrupted.

Overall, in MYXV, as in other complex DNA viruses, no common pathway to attenuation or virulence is observed, but a convergence for a phenotype that is compatible with multiple different genotypes and possibly involves complex epistatic interactions (Kerr 2012).

The genomic variability must be taken into account. On one hand, it can lead to very diverse clinical conditions, ranging from sudden death to clinical courses that take weeks and evolve to a slow death in a state of extreme cachexia and damage to the epidermis and genital tissues. On the other hand, aspects related to mutations in certain genes and variation between strains from different countries must be taken into account when designing molecular-based diagnostic methods.

1.6.4.1. Direct methods

MYXV identification can be carried out in the skin lesions (myxoma), eyelids and genital

mucosa, depending on the form and course of the disease and the strain. Although these three matrices are ideal for the cutaneous forms, MYXV can also be detected in internal organs (lungs, liver, spleen, kidney, etc) (OIE 2020).

Electron microscopy

Negative-staining electron microscopy (nsEM) can be applied using many biological samples such as myxomas, eyelids, and conjunctival and nasal swabs, among others. The drop method gives results in 1 hour since poxvirus are easily and specific to identify (OIE 2020). There are numerous protocols for sample preparation, which will not be explained here.

Histopathology

The lesions found in classic myxomatosis are very typical in the microscopic examination, namely the accumulation of mucin in the dermal connective tissue matrix with cellular dispersion (Marcato and Rosmini 1986). Infiltration by granulocytes and enlarged, stellate, reticuloendothelial cells with a large nucleus and abundant cytoplasm, called "myxoma cells" are observations suggestive of the disease (OIE 2020). The destruction of the endothelium of small vessels leads to micro haemorrhages. The replication of the myxoma cells in the spleen and lymph nodes causes a marked loss/depletion of lymphocytes from both B-cell and T-cell zones (OIE 2020). Viral spreading throughout the body causes genital and visceral lesions, due to congestion with vascular damage and oedema (OIE 2020).
For investigation purposes, many techniques of immunofluorescence and immunohistochemistry can be applied to understand the virus pathophysiology and tissue distribution and damage, namely using monoclonal antibodies made available by the International Reference Laboratory (Italy).

In-vitro and in-vivo virus isolation

Many cell lines are permissive to MYXV namely the RK-13 (rabbit kidney) and SIRC (Statens Serum Institut rabbit cornea), but also Vero (African green monkey kidney) and BGMK (buffalo green monkey kidney). Primary cell lines of rabbit kidney or fibroblasts can also be used. Poxviruses produce a characteristic cytopathic effect (CPE) (Joubert 1973), typically 24-48h after infection or up to 7 days, depending on the strain. According to the strain, the CPE can vary from well-defined voluminous masses of rounded cells, which proliferate and pile up (Joubert 1973), similar to CPE of Shope Fibroma virus or the formation of syncytia

with 2 to 100 nuclei (Joubert 1973). The confirmation of the presence of MYXV can be performed with immunofluorescence, directly on the plate of infected cells.

MYXV can also be isolated and cultured in the chorioallantoic membrane of embryonated chicken eggs inoculated on the 11th day of incubation. After 3 days, specific pocks are observed in the chorioallantoic membrane under the optical microscope.

Nowadays, rabbit intradermal inoculation is not used as a diagnostic tool. However, it is the only way to characterise the pathogenicity (virulence grade, classical or amyxomatous forms) of the strains. Typically, the animal is inoculated intradermally in the internal surface of the ear (0.1 to 0.2 mL) or the dorso-lumbar region. Different inoculum serial dilutions can be injected into the same animal at different sites, allowing the determination of the 50% infective dose (ID50). After 2-5 days, the primary lesion appears followed by conjunctivitis. If the animal survives, a robust immune response is detected 15 days after (OIE 2020).

Antigen detection

Agar gel immunodiffusion (AGID) (Sobey et al. 1966) qualitatively detects antigen or antibodies within 24 hours. The agar plates preparation and analyses of results are transversal with/to other AGID tests. Up to three lines can appear in the myxoma positive reactions indicating the presence of antigen. One line appears in the case of heterologous reactions with SFV (OIE 2020).

The direct immunofluorescence test (dFT) can be used to detect the antigen in tissue cryosections or in-vitro infected cells using fluorescein-conjugated anti-MYXV serum or MAbs. Alternatively, Indirect fluorescent antibody tests (iFT) can be used in cell cultures or tissue sections revealing intracytoplasmic multiplication of the virus. Other techniques such as the immunoperoxidase monolayer assay (IPMA) are also described, for which the protocol can be found in the OIE Terrestrial Manual (OIE 2020).

Molecular methods – DNA detection

The polymerase chain reaction (PCR) is the typical method for nucleic acid detection with many conventional (Cavadini et al. 2010; Dalton et al. 2019) and real-time PCR methods (Belsham et al. 2010; M.D. Duarte et al. 2014) available. The PCR and the PCR-RFLPs (restriction fragment length polymorphism) can also be designed to detect vaccine strains or natural recombinant strains (Cavadini et al. 2010; Camus-Bouclainville et al. 2011; Dalton et al. 2019).

Sequencing and full genome sequencing is increasingly a technique of choice that allows, on one hand, the definitive diagnosis and, on the other hand, the genomic characterization of the viral strain. Other techniques, such as those based on SYBR/Evagreen systems may present advantages in detecting single-nucleotide polymorphisms and cheap and easy multiplex systems.

1.6.4.2. Indirect methods

Specific antibodies are detected after-acquired passive immunity or active immunity following vaccination or natural infection, with the production of IgM and IgG classes (Kerr 1997). After recovery from infection with high virulent strains, the antibody titres are the highest, compared to the very low titres after vaccination or infection with low pathogenic MYXV strains (OIE 2020). IgM appears 5-6 days post-infection persisting up to 40 days, whereas IgG peas at 20-30 days, remain positive in naturally infected animals, for at least 2 years (OIE 2020). The cell-mediated immunity is more relevant than serum antibodies to the protection of rabbits (OIE 2020).

The detection of serum anti-MYXV antibodies can be achieved by the traditional agar gel immunodiffusion, seroneutralization test, complement fixation test (CFT) or by enzyme-linked immunosorbent assays (ELISAs). Due to poor sensitivity, CFT is no longer used is the ELISA the most used test.

Currently, for commercial and research purposes ELISA are available, namely indirect ELISAs (iELISA) with antigen directly coated to the solid phase (Kerr 1997; Gelfi et al. 1999), with full protocols available in the OIE Terrestrial manual. The OIE Reference Laboratory for Myxomatosis, developed and made available a competitive ELISA (cELISA), based on the use of a MAb (1E5) that specifically recognises the MYXV immunodominant envelope protein (IMV – open reading frame M071L) that detects antibodies in both rabbits and hares. This cELISA detects all the anti MYXV immunoglobulin classes present in the serum to the detriment of other ELISAs that use protein A as a secondary, that only detect IgG. The cELISA has higher specificity than iELISA (OIE 2020).

The OIE Terrestrial Manual also provides a protocol for preparing reagents and for performing AGID and IFAT techniques (https://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/3.07.01_MYXO.pdf).

1.7. Herpesviruses relevant in leporids

1.7.1. Emergence and spatiotemporal distribution

Herpesviruses are highly disseminated in nature with most animal species have yielded at least one herpesvirus infection (Pellett and Roizman 2013). Historically, herpesviruses have been classified based on the architecture of the virion and, in the late 1970s, before viral DNA and amino acid sequences were known, they were classified into a single family (Pellett and Roizman 2013). The virion structure (Pellett and Roizman 2013) consists of (i) a core containing a linear double-stranded DNA (dsDNA, ranging from 124–295 kb in length), (ii) an icosahedral capsid with approximately 125 nm in diameter containing 161 capsomeres with a hole running down their long axis, (iii) one capsomeric structure that serves as the portal for packaging and release of the viral genome - the complex of the core and capsid is the nucleocapsid, (iv) the tegument - an amorphous appearing, sometimes asymmetric material that surrounds the nucleocapsid and, (v) an envelope containing viral glycoprotein spikes on its surface. The availability of extensive nucleotide sequences (Davison et al. 2009; Pellett et al. 2012) allowed the establishment of a new taxonomic order, the *Herpesvirales* comprising three families: *Herpesviridae* – containing herpesviruses of mammals, birds, and reptiles, *Alloherpesviridae* – containing herpesviruses of fish and amphibians, and the *Malacoherpesviridae* – with the herpesviruses of bivalves.

Some herpesviruses have a wide host cell range, rapidly multiplying and destroying the infected cells (e.g., HSV1, HSV2), while others have a narrow host cell range (EBV, HHV6) or a long replication cycle (HCVM) (Pellett and Roizman 2013).

In general, this family show four significant biological properties (Pellett and Roizman 2013): (i) a large array of enzymes involved in nucleic acid metabolism (e.g., thymidine kinase, thymidylate synthetase, dUTPase, ribonucleotide reductase), DNA synthesis (e.g., DNA polymerase, helicase, primase), and processing of proteins (e.g., protein kinases), (ii) gene transcription, synthesis of viral DNA, and nucleocapsid assembly occurring in the nucleus. In most virions a part of the tegument is acquired in the cytoplasm, (iii) lytic infection leads to the destruction of infected cells and (iv) cellular latency as a mechanism for lifelong persistence in their hosts.

Due to the use of degenerate polymerase chain reaction (PCR) primers, that target highly conserved regions in core genes, such as the DNA polymerase, and the improvement of sequencing methods, there was an explosion of herpesviruses descriptions (Rose et al. 1998).

When mature, the virion size ranges from 120 to 260 nm according to thickness of tegument

and the state of the envelope (Roizmanm and Furlong 1974).

According to several studies (Johannsen et al. 2004; Kattenhorn et al. 2004; Varnum et al. 2004; Michael et al. 2006; O'Connor and Kedes 2006), it was estimated a total of 24 to 71 viral proteins in the virions, being 4-7 located in the nucleocapsid, 9-20 in the tegument, 4-19 in the envelope and a non-determined number with unknown location. The glycoprotein gene is so important and stable that a single virion contains approximately 800 copies of Glycoprotein B (Pellett and Roizman 2013).

The core of a mature virion contains a single molecule of dsDNA packed in a torus-form, so tightly that the internal volume of the capsid is approximately the cylindrical volume of the genome (Falke et al. 1959; Furlong et al. 1972). The capsid with100nm of diameter, contains 161 capsomers (10 hexons and 11 pentons), a portal complex with a capsid triangulation (T=16), preserved in all herpesviruses (Booy et al. 1996; Davison et al. 2005; Liu and Zhou 2007). The non-enveloped capsids detected in infected cells may present different forms, namely, A (capsids without core), B (capsids containing the assembly scaffold without genome) and C (capsids containing genome without scaffold) (Gibson 1996).

The proteinaceous structure between the nucleocapsid and the envelope is called tegument, sometimes distributed asymmetrically with variable thickness depending on the location of the virion within the infected cell (Pellett and Roizman 2013). In general, the tegument is thicker in the virions accumulating in cytoplasmic vacuoles than in those accumulating in the perinuclear space (Falke et al. 1959). This variability is more determined by the virus than by the host (McCombs et al. 1971). The tegument proteins closer to the nucleocapsid (inner tegument) are acquired in the nucleus and by interactions with envelope glycoproteins (Mettenleiter 2006). The subsequent components of the tegument are orderly incorporated during the path through the cytoplasm (Mettenleiter 2006). The tegument proteins are more important during the early phase of infection, by managing the cell environment, such as by shutting down host protein synthesis, inhibiting infection-triggered cell defences, and stimulating viral gene expression (Pellett and Roizman 2013).

The envelope derives from patches of altered nuclear membrane being mainly constituted by viral glycoproteins, which number and relative amounts vary among herpesviruses (Falke et al. 1959; Armstrong et al. 1961).

Within the herpesviruses, the linear double-stranded viral genome varies in length between 124 and 295 kbp, circularizing immediately after entering the nuclei of infected cells (Pellett and Roizman 2013). The genome contains terminal and internal repeated sequences, varying in copy number, and non-essential sequences that can be lost or duplicated during passage in cell culture. These genomic modifications lead to intraspecies variations that can exceed 10 kbp in length.

The genome of Herpesviridae members encodes between 70 (the smallest) and 200 (the largest) proteins (Pellett and Roizman 2013).

Herpesvirus genomes can be divided into six groups designated from A to F (Pellett and Roizman 2013), as follows:

A – Exemplified by HHV6, where a large sequence from one terminus is directly repeated at the other terminus;

B – Exemplified by herpesvirus saimiri (SaHV-2), where the terminal sequence is directly repeated numerous times at both termini, with a variable number of repeats at the termini;

C – Exemplified by EBV, where the number of direct terminal repeats is smaller and can harbour other direct sequence arrays that subdivide the unique (or quasiunique) sequences of the genome into several well-delineated stretches;

D – Exemplified by VZV, where one terminus is repeated in an inverted orientation internally. The domain consisting of the stretch of unique sequences flanked by inverted repeats (Small or S component) can invert relative to the remaining sequences (Large or L component);

E – Exemplified by HSV and HCMV, where the sequences from both termini are repeated in an inverted orientation and juxtaposed internally, dividing the genomes into two components, each of which consists of unique sequences flanked by unrelated pairs of inverted repeats;

F – Exemplified by tupaia herpesvirus 1 (TuHV-1), where the terminal sequences are not identical and are not repeated either directly or in an inverted orientation.

Two key characteristics are found in herpesvirus: *i)* the gene products frequently have multiple functions that may, or not, be related, and *ii)* most of these genes are not dispensable for viral replication or reactivation from the latent state, despite no virus gene is indispensable during the establishment of latency. Genes that are necessary for virus replication in cultured cells are sometimes referred to as "essential" or "fundamental," and dispensable genes as "nonessential" or "accessory" (Pellett and Roizman 2013).

The replication cycle occurs in three major phases: initiation of infection, lytic replication and latency (Pellett and Roizman 2013). During the initial steps of infection, several events take place namely, binding to the cell receptor, fusion of the viral membrane with the plasma membrane or after endocytosis, management of intrinsic response by tegument proteins, transport of nucleocapsid and tegument associated IE-activators to the nucleus, injection of viral genome through the nuclear pores and genome chromatinization, and initial interactions with transcriptional machinery (Pellett and Roizman 2013).

Then occurs a biological decision at cell level to follow either the lytic or latent pathway, as well, after the initial infection and latency, reactivation of a lytic state (Pellett and Roizman 2013).

During the lytic replication, a regulated cascade of lytic gene expression occurs, management of the host cell (metabolism, protein synthesis and stability, cell cycle, intrinsic and innate defences), management of adaptive immune response, replication of virus genome, virus assembly and egress and transmission to other cells and hosts (Pellett and Roizman 2013). When the virus enters the latent pathway, restriction of lytic gene expression and expression of latency genes that manage the cell and host defences and maintains the virus genome in the infected cells occurs (Pellett and Roizman 2013).

1.7.2. Pathophysiology

In rabbits, *Herpesviridae* is a poorly studied family, with most studies dating before the XXI century. Rabbit species have been used as an experimental model for human herpes simplex virus (HSV), genus *simplexvirus*, alphaherpesvirus. However, there are reports of natural disease in rabbits that led to fatal encephalitis, after contact with infected humans, despite this natural cross-infection is infrequent (Weissenbock et al. 1997; Grest et al. 2002; Muller et al. 2009; Kolodziejek and Huemer 2010)

Rabbits infected with HSV present severe signs of central nervous system dysfunction, myoclonic seizures, opisthotonus, lymphopenia, monocytosis, an increase of creatine phosphokinase and total proteins (Muller et al. 2009). The presumptive diagnosis is achieved by histopathology, being observed severe, diffuse, nonsuppurative meningoencephalitis and a few large, eosinophilic, intranuclear inclusion bodies in neurons. The herpesvirus DNA can be confirmed by *in situ* hybridization, PCR, among others, in nuclei of glial cells, lymphocytes, and neurons (Gruber et al. 2009).

Three gammaherpesviruses naturally occurring in rabbits have been described (Pellett and Roizman 2013), formally named as leporid gammaherpesvirus 1 (LeHV-1), leporid gammaherpesvirus 2 (LeHV-2) and leporid gammaherpesvirus 3 (LeHV-3), although these classifications are provisional (Jin, Löhr, et al. 2008).

LeHV-1 is found in cottontail rabbit (*Sylvilagus* spp.) causing no disease in domestic rabbits (Jin, Löhr, et al. 2008). LeHV-2 (informally named Herpesvirus cuniculli) causes asymptomatic infection of domestic rabbit (Jin et al. 2008) and LeHV-3 (informally named Herpesvirus sylvilagus) is endemic in Eastern cottontail rabbits (Sylvilagus floridanus) causing tumorlike lesions in lymph nodes, kidney, spleen and liver (Medveczky 1999).

In 1991, a new herpesvirus was described in Alaska and Canada (Swan et al. 1991; Onderka et al. 1992; Erlach and Ildfell 2008; Brash et al. 2010), formally designated as Leporid alphaherpesvirus 4 (LeHV-4), genus *Simplexvirus*, subfamily *Alphaherpesvirinae*, closely related to bovine herpesvirus-2, and also to HSV-1 and HSV-2 (Jin, Löhr, et al. 2008). This

virus proved highly pathogenic for domestic rabbits and reports are limited to commercial rabbitries. Experimental infections observed systemic infection and caused severe clinical disease and necrosis in the spleen and lymph nodes (Jin, Löhr, et al. 2008).

In animals infected by LeHV-4, the primary lesions are conjunctivitis and periocular swelling, multifocal hemorrhagic/ ulcerative dermatitis on the face and dorsum (Onderka et al. 1992) and then progressive weakness, anorexia, respiratory distress, and abortion and can also occur sudden death (Jin, Valentine, et al. 2008). Cardiovascular and respiratory failure leads to death (Swan et al. 1991).

Histopathology reveals massive necrosis and fibrin deposition within spleen red pulp (Jin, Valentine, et al. 2008) with large eosinophilic intranuclear inclusion bodies identifiable in sections of skin, spleen, and lung (Onderka et al. 1992). Haemorrhagic dermatitis and panniculitis, epidermal microvesicular degeneration, dermal and subcutaneous vascular necrosis and thrombosis may also be observed. Other less frequent observations are haemorrhagic necrosis of the myocardium with rare intranuclear inclusions within stromal cells, multifocal pulmonary haemorrhage, and haemorrhage with erythrophagocytosis in lymph nodes sinus (Jin, Valentine, et al. 2008).

1.7.3. Diagnosis

As described before, although in the case of rabbit herpesviruses the clinical signs are not pathognomonic, a presumptive but no definitive diagnosis can be made based on histopathological lesions, particularly by the observation of intranuclear characteristic inclusion bodies.

1.7.3.1. Direct methods

Electron microscopy

The electron microscopy can be made either after viral isolation in cell culture, directly from the tissues infected or from the lesions fluid (e.g. vesicles). In any case, the morphology of the virus is unique and confers a final diagnosis when observed. The vesicle lesions should be analysed, ideally within the first 24h after their appearance, since once the lesion begins to crust, the test sensitivity declines (Singh et al. 2005).

Histopathology

The necropsy findings and subsequent histopathology are variable according to each herpesvirus, particularly depending on the subfamily to which they belong. For example, the leporid gammaherpesvirus 2 (LeHV-2) is associated with subclinical encephalitis in infected New Zealand white rabbits (Zygraich and Berge 1972), while the leporid gammaherpesvirus 3 (LeHV-3) causes lymphoproliferative disease and tumour-like lesions in the lymph nodes, kidneys, spleen, and liver (Hinze 1971; Hesselton et al. 1988).

The leporid alphaherpesvirus 4 has been associated with haemorrhagic dermatitis, splenic necrosis, hepatic necrosis, multifocal pulmonary haemorrhage and oedema. Distinctive glassy eosinophilic herpetic intranuclear inclusion bodies have been observed in the skin fibroblasts and mesenchymal cells of the spleen and lung (Jin, Löhr, et al. 2008; Brash et al. 2010).

In-vitro and in-vivo virus isolation

LeHV-2 was successfully grown in PRK monolayers and cell cultures such as RK-13 and SIRC continuous lines exhibiting Cowdry type A intranuclear inclusions, as well as in primary tissues such as whole embryo, kidney, lung, salivary gland, and testis, which proved to be useful for virus isolation.

LeHV-4 was able to grow in kidney cell cultures inoculated with pooled lung, liver, spleen, kidney, and skin samples, showing cytopathic changes by five days postinoculation (Onderka et al. 1992).

In general, the specimen may be adsorbed onto the cell monolayer after removal of the medium 30 to 60 min at 37°C, facilitating direct contact of viral particles with the cells and enhancing infectivity, increasing both the number of isolation and the speed with which they are recovered (Singh et al. 2005).

To reduce the viral isolation time, centrifugation-enhanced (shell vial) culture methods can be used (Wiedbrauk and Johnston 1993), reducing viral isolation times from 1-7 days to 16-48h (Singh et al. 2005) in the case of HSV. This method proved rapid and specific, but slightly less sensitive than traditional tube cultures (Johnston and Siegel 1990).

Antigen detection

The antigen can be defected by direct fluorescent antibody (DFA), a relatively fast but highly specific and sensitive method, particularly in the initial phase of infections (Wiedbrauk and

Johnston 1993). A slide directly prepared by the clinician, or ideally prepared at the laboratory using a cytospin method or a swab specimen can be used. The big limitation of this method is the need for fluorescein labelled antibodies, often not available to wildlife viruses, namely to recently discovered viruses. A positive test appears as a typical pattern of green fluorescence in the nucleus or cytoplasm of the infected cells.

Molecular methods

Viral DNA can be detected by hybridization techniques using radiolabelled or biotinylated probes (Langenberg et al. 1988), having been largely superseded by more sensitive and less laborious procedures such as the polymerase chain reaction (PCR).

In 1996, the first PCR for herpesvirus detection which amplified a region of DNA-directed DNA polymerase (EC 2.7.7.7) using degenerate primers in a nested format (Devanter et al. 1996) was published. This system targets sequences coding for highly conserved amino acid motifs (Wilks et al. 1989). The first PCR round uses a set of three primers (namely two forward DFA, ILK, and one reverse KG1) and the second round a set of two primers (forward TGV and reverse IYG). The Secondary PCR products range in size from approximately 215 to 315 bp (distinct for different human and animal herpesvirus). Several adaptations of this method were published, for example, using deoxyinosine substituted primers or consensus-degenerate hybrid oligonucleotide primers. Some limitations of this methodologies are the difficulty in detecting co-infections (e.g. in pigs infected with PLHV-1, PLHV-2 and PLHV-3, the last is not amplified) and the short size of amplified sequences, often not sufficient for the construction of phylogenetic trees revealing acceptable probabilities for all clades (Prepens et al. 2007).

Recently, new methods were described to overcome these shortcomings, namely a method that amplifies DPOL in the presence of an additional oligonucleotide modified by the introduction of locked nucleic acids (LNA) and also amplifying the less conserved glycoprotein B (gB) gene with degenerate primers of limited detection capacity i.e. genus-specific primers (Prepens et al. 2007).

1.7.3.2. Indirect methods

These techniques are particularly useful in identifying asymptomatic carriers of infection and performing seroepidemiological studies in wildlife or captive populations. In the actuall context of herpesvirus of leporids, these techniques are not useful due to the absence of fully reliable methods.

1.8. Research objectives and organization

Taking into account the previously described state-of-the-art, an exhaustive multidisciplinary approach was attempted to help assess and characterize the various threats that presently affect the conservation status of wild leporids in the Iberian Peninsula, while finding means to control such hazards.

During these scientific studies, within the framework of a PhD in co-tutorship between the University of Lisbon (Veterinary Sciences, special field of Animal Health) and the University of Oviedo (Molecular and Cellular Biology), there was a need to develop clinical (scientific publication 1), cellular (scientific publication 2) and molecular methods (scientific publications 4 and 5) of to be able to carry out the various scientific studies and to contribute to the investigation on these subjects by various research groups. The molecular first-line results obtained led us to reason upon their meaning, sharing these reflections with the scientific community (scientific publication 3). This sharing led to the fact that the various works presented here with a molecular core were robustly supported by data from other scientific areas.

At the moment of this PhD program proposal (beginning of 2018), only the study of the viral hemorrhagic disease of the wild rabbit was contemplated. The other two threats of viral aetiology addressed here (Myxomatosis in the Iberian Hare and LeHV-5) were not even described, having later been included in the doctoral program due to their scientific and sanitary importance.

Although the PhD includes studies that required complex and time-consuming programming (such as the RHDV2 research study in badgers or the Iberian hare, molecular diagnosis methods or wild rabbit vaccination study that represented studies lasting more than two years), the other studies were mostly the result of unscheduled discoveries during the clinical and pathological investigations of Project +Coelho, constituting as a whole, an assessment of the impact of diseases of viral aetiology in wild leporids.

Consequently, this document presents a collection of scientific works, which address issues such as the emergence, pathophysiology, prophylaxis or diagnosis of three threats of wild leporidae in Portugal: RHDV2, MYXV and LeHV-5, organized functionally into three chapters: a chapter that describes new methods developed of clinical, cellular or molecular nature, also including a reflection on the application of molecular data in the context of research in animal health. This is followed by a chapter including research articles in RHDV2 and then a chapter that includes the research outcomes on MYXV and LeHV-5.

All the content constituent of the scientific publications is already published in peer-reviewed International Journals and presented using the published layout.

Clinical, cellular and molecular methods

This chapter describes new methods developed for diagnosis and research namely an optimized method of leporid blood collection, a method for establishing primary leporid fibroblast cultures for virus isolation, a Quadruplex PCR method for diagnosis and differentiation of MYXV strains, and the first qPCR method for diagnosis of LeHV-5.

Chapter 2 also includes considerations about the interpretation of molecular biology-based results.
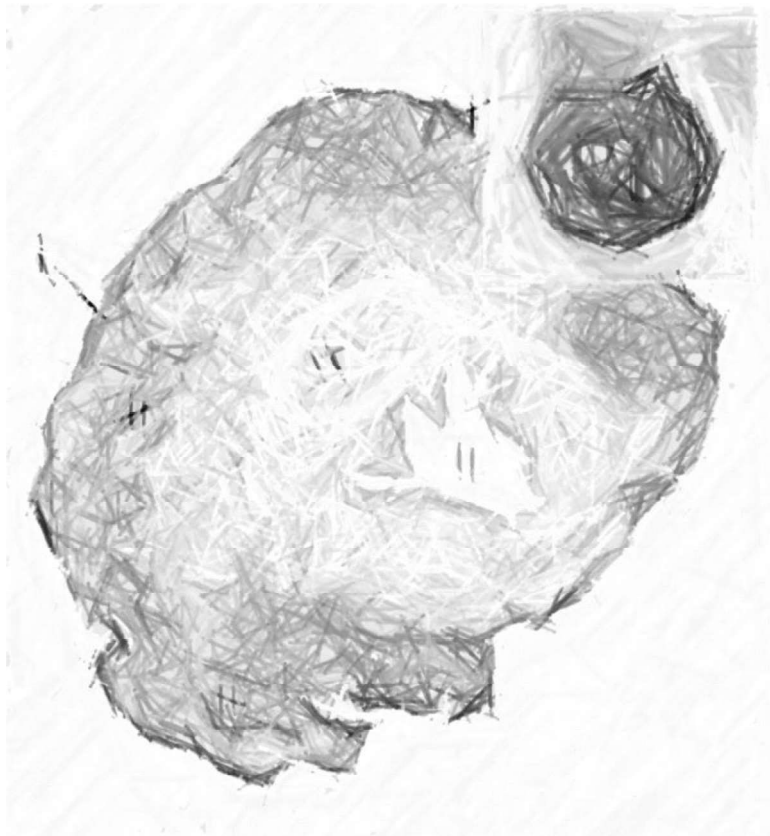
How to collect blood samples from wild leporids?

Scientific publication 1

Blood collection from the external jugular vein of *Oryctolagus cuniculus algirus* sedated with midazolam: live sampling of a subspecies at risk

■ ■

The external jugular vein provides an easy and safe route to collect blood from wild leporids

Blood collection from the external jugular vein of Oryctolagus cuniculus algirus sedated with midazolam: live sampling of a subspecies at risk

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Blood collection from the external jugular vein of *Oryctolagus cuniculus algirus* sedated with midazolam: live sampling of a subspecies at risk

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In the last decades, the European wild rabbit, particularly the *Oryctolagus cuniculus algirus*, a keystone species in the Iberian Peninsula ecosystems, declined severely, raising concerns from the wildlife authorities. The hunting calendar in Portugal limits sampling collection to a narrow window of few months annually. Nevertheless, governmental wildlife protection laws allow live rabbit sampling for population and sanitary evaluations. The aim of this study is to adjust blood collection protocols from the external jugular vein (EJV) described for domestic rabbits to the wild rabbit. Collection of peripheral blood is problematic in the wild rabbit given its small body size and the reduced calibre of vessels but mostly its nervous disposition and fragility. We describe in detail a procedure for EJV blood collection in 30 wild rabbits after sedation with midazolam. Emphasis is given to protocol adjustments for wild rabbit. Heart rate, respiratory rate and body temperature were assessed before sedation, after sedation but before collection and after blood collection. Sedation onset took on mean (SD) 8 ± 2 min. The technique allowed the collection at least 1 ml of blood, a satisfactory volume for routine laboratory testing. The differences observed in heart and respiratory rates before and after blood collection were not statistically significant, indicating that no cardiorespiratory interference occurred due to venepuncture. Recovery from sedation took on mean (SD) 17 ± 2 min. All animals were set free during the first hour after blood collection. This work aims to demonstrate that blood collection under sedation is a safe and feasible procedure in wild rabbits when practiced by experienced veterinarians. At no time, whatsoever, was the physiological homeostasis at risk and no injuries were inflicted on the animals. To our knowledge this report constitutes the first guided description of blood collection from the EJV in sedated *O. c. algirus* and the first collection of physiological parameters measured under different conditions.

Keywords: blood collection, external jugular vein, *Oryctolagus cuniculus*, sampling live animals, sanitary surveillance, venepuncture, wild rabbit

The European wild rabbit *Oryctolagus cuniculus* (Linnaeus 1758) is a small herbivorous mammal belonging to the *Leporidae* family of the *Lagomorpha* order whose main morphologic, biologic and physiologic characteristics are summarized in Table 1.

The wild rabbit is a keystone species in Mediterranean ecosystems of Iberian Peninsula in which the preservation of many vulnerable and threatened mammals and bird species depend on. Moreover, the rabbit has an important role in habitat modulation, due to its digging activity, selective plant ingestion, seed dispersal and soil fertilization, supporting the growth of typical vegetation (Willott et al. 2000, Virgós et al. 2005) and indirectly contributing for the diversity of soil invertebrate community.

In 2008, the International Union for Conservation of Nature (IUCN) rated *O. cuniculus* species as near threatened (NT), following its progressive decline in Europe since 1950, reaching 95% reductions. The most important factors behind the wild rabbit decline are the loss of habitat due to fragmentation and intensive farming, anthropogenic activities (Ward 2005) and the emergence of viral diseases in the 20th century (Delibes et al. 2000). Confinement of

Table 1. Morphological, biological and physiological data from wild rabbits.

Parameters		Available information	References
Morphological	head–body length (adults)	34–50 mm	Macdonald and Barrett 1993
	rabbit fur	mixed brown-grey fur coat, of yellowish tones in the occipital region and legs, and whitish belly and white inner short tail	La Fuente 1993, Gálvez-Bravo 2017
	others	black lateral eyes and long ears though smaller than those of hares. Strong hind legs are more developed than the front ones, allowing them to sprint in jumps and zigzags	
Biological	reproduction season	annual (depending of clime and resource availability)	Bell and Webb 1991
	litter size	3–6 kittens, which leave the warren in under a month$	Gibb and Williams 1990
	sexual maturity	♂ 4 months; ♀ 3.5 months	Macdonald and Barrett 1993
	lifespan	♂ max 8.7 years; ♀ max 7.7 years	Macdonald and Barrett 1993, Von Holst et al. 1999
Physiological	body temperature	38.5–39.5°C (101.3–103.1°F)	Patrick et al. 1994
	fasting metabolic rate	750 kcal m^{-2} body surface area	Patrick et al. 1994
	respiratory rate	30–60 breaths per minute*	O'Malley 2005
	heart rate	180–250 per minute	O'Malley 2005
	water intake per day	50–150 ml kg^{-1}	Cizek 1961
	urine	alkaline (pH 7.6–8.8), specific density: 1.003–1.036, normally cloudy	Patrick et al. 1994

$ 3/4 of young rabbits are killed by predators before they establish a territory (Gibb 1990, Angulo 2004). Annual mortality of general population was 30% in a studied island (Macdonald and Barrett 1993).
* At rest, rabbit breath mainly using muscular contractions of the diaphragm, with reduced movements of the rib cage. Artificial respiration can be provided moving the rabbit from a head-up to head-down position 45 times per minute (Patrick et al. 1994).

O. cuniculus algirus subspecies to a few restricted geographic locations (including the south–west of the Iberian Peninsula, the Atlantic islands and Morocco) makes its surveillance and recovery even more urgent.

Reverting the recent abrupt decrease of wild rabbit populations due to highly virulent pathogens, such as rabbit haemorrhagic disease virus (RHDV) and myxoma virus (MYXV), requires disease surveillance programmes to assist and support sustainable management of this wild species. Like Myxomatosis, Rabbit haemorrhagic disease is endemic in some countries, including Portugal, occurring in the mainland (Abrantes et al. 2013), autonomous regions (Duarte et al. 2015, Carvalho et al. 2017) and the islands such as the small Berlengas archipelago (Abade dos Santos et al. 2017). Regarding our team, the need for the development a blood collection protocol emerged/was identified during a study conducted in the Berlengas archipelago to investigate haemoparasites and viruses in the local rabbits (Abade dos Santos et al. 2017).

Due to the difficulty in capturing and sampling live specimens without causing great stress, monitoring of wild rabbit populations is usually carried out in biological samples collected from legally hunted rabbits' cadavers. Collections are therefore limited to the hunting season, or dependent on dead animals found in passive surveillance. Furthermore, in areas where the rabbit populations are extremely low, hunting is often suspended to accelerate recovery, precluding sampling.

However, longitudinal serological surveys have proven crucial to evaluate wild rabbit population's immune status as well as to provide a dynamic view on the pathogen–host evolution and population equilibrium. Blood samples allow not only serologic investigations but also culture and/or direct examination for bacteria, viruses and some parasites.

In domestic rabbits, selection of the blood collection method depends on the animals' behaviour, amount of blood needed, frequency of sampling and whether the animal's survival is required or not. Blood can be withdrawn from the marginal ear veins, central ear artery, cephalic vein and lateral saphenous vein. Domestic rabbits have also large paired external jugular veins (EJV, vena jugularis externa), which are often the preferred venepuncture site under sedation or when a larger amount of blood is required (Nelson et al. 2010). The external jugular vein is formed behind the angle of the mandible, passing backward in a superficial position to the superior thoracic aperture (Nelson et al. 2010), and the work zone for blood sampling is the ventral surface of the neck.

Despite the animal's clinical condition must always be considered, a general rule of thumb is that it is safe to take up a maximum of 1 ml per 100 g bodyweight (Ramer et al. 1999), i.e. 1% (v/w) of bodyweight. All non-terminal blood collection carried out without fluids replacement is limited up to 10% of total circulating blood volume in healthy, normal, adult animals on a single occasion and collection may be repeated only each three to four week. Deep sedation of rabbits is sometimes required for adequate restraint, evaluation and minor procedures (Cantwell 2001).

Contrarily to domestic rabbits, blood sampling in wild rabbits poses serious difficulties due to the extremely nervous nature of the species and innate instinctual fear of humans, causing great stress that may lead to the onset of diseases or even to sudden death (Mullan and Saunders 2018). In wild rabbits, it is very important that handling stress and pain inflicted during blood collection are reduced to a minimum. In addition, the small size of wild rabbit and consequent reduced vessel diameter further complicates peripheral blood collection. The cephalic veins are difficult to locate and hold off and while the lateral saphenous vein is easier to locate it has a very small diameter, making the collection of an adequate blood sample difficult (Graham 2006). Furthermore, hematoma, bruising, vessel thrombosis and skin sloughing

can also occur due to the vessels reduced diameter and the use of ear veins is not recommended in this species (Graham 2006). In juveniles, the blood sampling technique is even harder to perform.

When collecting a blood sample from wild rabbits, cautions must be taken regarding the animal as well as the handler. Clinical fragilities inherent to wild rabbits include risk of heart attack, dehydration and bone fracture. The knowledge of the physiological parameters of this species is critical (Table 1). As to the handler, when the animal is picked up without the appropriate support of the body and hindquarters, their very sharp claws may inflict painful scratches which easily become infected (Varga 2014). Nevertheless, claws should not be cut off as they are important in rabbits' movements and digging ability. Bites can equally result in skin and soft tissue infection.

Several parenteral anaesthetic protocols for rabbits and rodents, either for research or in pet clinics, are described in literature (Cantwell 2001). Furthermore, many anaesthetics dosages referenced for the rabbit have been developed for use in the laboratory rabbit which is, generally, a robust animal that has been bred to be pathogen free (Borkowski and Karas 1999).

The available anaesthetic protocols, usually designed for clinical purposes, need to be adapted to wildlife/free-living animals. Benzodiazepines, including diazepam, midazolam and zolazepam, are muscle relaxants that act centrally, producing excellent relaxation, reducing muscle spasms and spasticity (Muir et al. 2000), while not inducing muscle paralysis (Flecknell 1987). Sedation induced by this group of drugs is very marked in rabbits and rodents (Flecknell 1987) despite not producing a true anaesthetic state, as awareness persists with relaxation even at high dosages, or any analgesic effect.

Midazolam causes minimal hemodynamic and respiratory changes and is often chosen over diazepam for its water solubility which allows mixing it with other water-soluble substances in a single syringe (Henke et al. 2005). It is also more effective that diazepam (Longley et al. 2008) and has a shorter duration of action (Flecknell 1984). For diagnostic procedures, the use of intramuscular or intravenous midazolam has been recommended due to its short acting sedation (Ramer et al. 1999).

To our knowledge, and from the published bibliography, collecting blood from the EJV from wild rabbits is not used routinely because requires experience, training and a solid knowledge of the anatomy and relative dimensions of the wild rabbit. On the other hand, this work somewhat demystifies the idea that sedation, handling and blood sample of large volumes in small mammals is risky in the field and reinforces the importance of longitudinal study of populations of small mammals, many of them at risk of extinction.

In this work, we describe an EJV blood collection procedure in wild rabbits, based on the protocol described by Nelson et al. (2010) and taking into account the idiosyncrasies of this wild species, under sedation with midazolam, which has proven to be a safe and effective technique for blood sampling in this species under field conditions and allows the collection of a substantially high volume of blood without clotting or haemolysis.

Material and methods

Animals

A total of thirty wild rabbits (12 males and 18 females; 16 adults and 14 juveniles) were analysed in this study. Pregnant females were not used. Animals were captured in Aldeia Gavinha (n = 14) and Quinta dos Penedinhos (n = 9), two reproduction centres for *Oryctolagos cuniculus algirus* near Lisbon, and in two hunting reserves in Alentejo, Herdade das Romeiras (n = 2) and Vila Nova de Mil Fontes (n = 5).

The study took place between December 2017 and February 2018 and was carried out within the scope of the Action Plan for the Control of the rabbit haemorrhagic disease (Dispatch no. 4757/2017 of 31 May from the Portuguese Ministry of Agriculture, Forests and Rural Development) and +Coelho project funded by Fundo Florestal Permanente.

No animals were sacrificed for the purposes of this specific study and all procedures were performed and monitored by veterinarians. The data of all animals will be available from the Dryad Digital Repository.

Capture method

The 23 wild rabbits bred in semi-captivity, originating from Lisbon, were caught with painless lever traps, using vegetables and fruit as bait. Traps were checked every 2 h during the day and every 7 h at night. After capture, the animals were kept at maximum 8 h at constant temperature (20°C) and humidity (65%) as well as in the recommended area per animal described in the literature (Buil et al. 2004) with good ventilation. The five freedom rules recommended by the World Veterinary Association (Seamer 1993), were followed as much as possible, namely water and food ad libitum, no infliction of unnecessary pain and stress, and release to nature as fast as possible. Animals originating from the same colony were maintained in groups with only one male per cage and separated according to age. All boxes and bags were used by a group and one animal respectively, on each day of procedure. At the end, boxes were washed with pressurized and warmed water and then sprayed with 5% sodium hypochlorite for decontamination and used after a minimum of two weeks. The bags were washed at 80°C. Animals from the same colony were kept in the same box during the procedure, after clinical evaluation to ensure that they were not sick. The issue of transmission of rabbit haemorrhagic disease virus (RHDV2) or myxomatosis was considered of minor importance since all animals used were vaccinated.

The five rabbits originating from the hunting reserve in Vila Nova de Milfontes were captured with ferrets, according to the Portuguese legislation (Decree Law no. 202/2004 of 18-08-2004, article 85) and kept in the same conditions. Between data assessments, animals were kept in suspended dark fabric bags, allowing them to keep calm and breathe normally.

Clinical evaluation

Animals were manipulated in situ. The animals were handled using clothing that covered the sleeves to minimize the risk of bite and scratch. Cotton gloves covered by nitrile or latex

gloves were used, the latter being changed between different animals.

The rabbits' physical condition was classified as physical status I, according to the American Society of Anaesthesiology criteria (ASA).

Animals were weighed (with a precision of 50 g) to calculate the sedative dose, and their physical condition and body length were assessed. Other morphological parameters (head, ear and tarsus length) were also registered. Given the good body condition of all animals, those weighing less than 900 g were considered juveniles and those with equal or greater weight considered adults.

Heart rate was measured with a pulse oximeter and ECG apparatus (monitor Cygnus 80E vet) designed for veterinary use. The pulse oximeter probe was placed on the lip and the ECG clips in the left and right anterior limbs and left posterior limb according to manufacturer's instructions. Shallow ECG clips were used to avoid skin damage and to minimize animal disturbance. A 70% ethanol spray was used to deflect the hair and remove the oil from the skin allowing full contact with the electrodes. Respiratory rate was determined by observation of the animals' thoracic movements and by auscultation with a stethoscope. Body temperature was measured with a digital thermometer (with a precision of $\pm 0.01°C$) on the rectal mucosa.

Parameters were taken before sedation (BS), after sedation and before collection (BC) and after collection (AC). Time of sedation onset and of recovery were registered.

Sedation protocol

Because wild rabbits are easily stressed and small, therefore more difficult to contain and bleed, both juveniles and adults were sedated. Sedation was performed by intramuscular (longissimus and iliocostalis muscles) administration of midazolam at a dosage of 1 mg kg^{-1}, a protocol that provides moderate sedation.

A specific antagonist, flumazenil, was available to reversed sedation if necessary at a dose of 0.01–0.1 mg kg^{-1} IV (Flecknell 1987, Gargiulo et al. 2012).

The method we used to contain the animal was adapted to the idiosyncrasies of wild rabbit protocol described by Nelson et al. (2010). Approaching the rabbit inside the transport boxes was made slowly and precisely. The handler grasped the rabbit through the skin of the back immediately behind the neck (Fig. 1A). A good area of skin was hold with the dominant hand to immobilize the animal allowing sedative administration.

After the onset of sedation, the handling approach was the same described above. With the non-dominant hand, the lumbar region of the animal is secured to prevent vertebral injuries and minimize limb movements (Fig. 1A). With a smooth and continuous movement, the animal's body was inverted to a lateral (Fig. 1B) and finally dorsal decubitus position without ever removing the support from the lumbar and cervical zones (Fig. 1C). The hind limbs of the animal were kept between the body and the medial face of the restrainers' arm (Fig. 1D).

Relaxed members (Fig. 1B) indicated that the animal was well restrained and stress-free. The pressure of the dominant hand on the skin of the back is never released so that the animal does not have the impetus to try to dodge. This pressure, as well as pressure under the abdomen and hind limbs, must be especially firm during venepuncture.

Venepuncture procedure

A rubber elastic (Fig. 1F) was placed caudally to the venipuncture site, precisely in the entrance of the chest, thus promoting venous distension of the EJV allowing a better visualization. The elastic was positioned by the person holding the animal, or by a third person, to apply pressure laterally, only on the selected EJV avoiding pressuring the trachea. The EJV veins appear blue in colour and are found around the caudal end of the mandible (Fig. 1E).

A sprayer with 70% ethanol was used to clean the skin and evidence EJV. Considering the size of the rabbits utilized in this study and their individual characteristics, needles with 25G × 5/8″ (0.5 × 16 mm) or 26G × 1/2″ (0.45 × 12 mm) were used as well as a 1mL syringe. No more than 3–4 mm of needle was inserted in the cephalocaudal direction (front to back) into the blood vessel, as recommended by Parasuraman et al. (2010), and blood slowly withdrawn to avoid the collapse of the vessels (Fig. 1F). To facilitate the insertion of the needle into the vein the needle can be folded about 5–10° using the needle cap. To facilitate/enable a better visualization of the EJV, or whenever blood sampling is to be repeated, clipping the fur on the area may be recommended (Fig. 1E). All animals used in this study were not sheared except the one used for demonstrating the procedure as shown in Fig. 1E.

Finger pressure was applied for 1–3 min after the needle's removal to stop bleeding (Parasuraman et al. 2010, Boyle 2016). After the procedure, the area was disinfected with a 5% chlorhexidine solution. To avoid unwanted cooling of the animals, we developed a thermostat-regulated infrared lamp heating box with an internal temperature of 20°C, where animals could recover their body temperature faster, whenever necessary (Fig. 2). Animals were released into their source colony when the effect of sedation was no longer evident.

For monitoring the animals' recovery, relying on individual identification, the rabbits originating from Aldeia Gavinha (District of Torres Vedras) were marked with an inactivated calcium carbonate solution at both their ear tips, on medial and lateral sides. This compound is inert to the animal's skin, allowing temporary marking visualization in both coloured and black and white photographs, disappearing over time.

For monitorization of a subset of wild rabbits (those caught at reproduction centers), night (with black light) and day monitoring cameras were installed, and the animals were observed for five days. Wheat and corn were scattered next to the cameras to attract the animals.

Genetic assessment

In order to assess the taxonomic status of the animals obtained during this study from the different sampling origins and confirm they belonged to the subspecies *O. c. algirus*, a genetic analysis was carried out, allowing the distinction from the subspecies *O. c. cuniculus* (including domestic breeds).

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Figure 1. Sequence of procedures for containment and presentation of the animal for blood collection. First approach (A), lateral decubitus (B), dorsal decubitus (C), presentation of the animal for blood collection (D), visualization of external jugular vein (E) and blood collection (F). The arrow indicates the beginning of the external jugular vein. Cranially at this point are the linguofacial and retromandibular veins.

During handling, a small ear biopsy was obtained from a subset of the captured rabbits (n = 12) and kept in 96% ethanol until further analysis. Additionally, samples from five domestic rabbits were obtained for comparative purposes. Genomic DNA was extracted using the EZNA DNA purification kit (Omega) following the manufacturer's guidelines. Two molecular markers were amplified via PCR to infer the taxonomic status of the analysed rabbits: the mitochondrial control region and the first intron of the coagulation factor IX (F9) gene (X chromosome). Obtained sequences were compared with those published in public databases for subspecies inference. The details of the protocol are not revealed in this study because they are not within its scope.

Statistical analysis

Initially, an exploratory analysis of the data was performed using syntax Proc univariate, Proc means and Proc freq of SAS 9.4 software (2013) in order to characterize the different variables included/considered in this study. The research of extreme outliers was investigated, whenever they were below the lower outer fence or above the upper outer fence. Extreme outliers were only found in the temperature measurement and were not removed due to small sample size and clustering. Values biologically non-logical (e.g. heart or

Figure 2. Recovery of body temperature in a box with infrared light heating regulated by thermostat.

respiratory rate outside the physiologically possible range) were also searched for but were not found. The data for the response variables (heart rate, respiratory rate and temperature) was assessed for normality by observing the QQ plots and running the Shapiro–Wilk normality test. The heart and respiratory rates showed no normal distribution ($p < 0.05$) so logarithmic transformation of these data was performed. To evaluate the evolution of the response variables over the three measurement moments: 1) before sedation (BS), 2) after sedation but prior venepuncture (BC) and 3) after venepuncture (AC), the SAS 9.4 PROC MIXED was used. A confidence interval of 95% (p-value <0.05 significant) was considered.

Results

Morphological parameters

The mean and standard deviations values for the morphological parameters collected from all animals (weight and body length, as well as head, ear and tarsus dimensions) are shown in Table 2. The p-values were obtained with t-test for equality of means after confirmation of equality of variances assumed by the Levene test.

Onset and recovery of sedation

The mean time for the onset of sedation occurred within 7.6 ± 1.9 min after intramuscular administration of midazolam, as determined by the reaction to handling (calmer and more permissive). There was no reaction to sedative administration.

On mean $\pm$ SD, 17.3 ± 2.2 min after sedation, all animals had recovered fully from sedation without the need of antagonist use. Animals were considered recovered from sedation when there was an escape reaction to handling. Altogether, restraint, disinfection and blood collection procedure took an mean (SD) 45 ± 20.5 s.

Blood sampling

The sedation and handling method described here allowed the collection of 1 ml of blood in all of animals, without clot formation. There were no sudden movements of animals that jeopardized their integrity and no major haemorrhage or hematomas were seen. The visualization of the vein was performed with relative ease and the collection performed without need of repetition of the puncture.

Physiological parameters variation

t-test for comparing group means did not reveal differences between the juvenile and adult physiological parameters, except for respiratory rate after venepuncture ($p < 0.05$). Because the value of F was smaller and significantly superior to 0.05, equal variance of data between the two groups may be assumed. So, data from the two groups of animals (juvenile and adults) were analysed together for greater statistical robustness.

The heart rate, respiratory rate and temperature were generally higher in the group of animals captured with ferrets (Table 3), although the statistical significance was not tested given the of the subsample. As expected, heart rate decreased with sedation in mean 45 beats per minute ($p < 0.0001$) but remained stable during the venepuncture procedure. On mean, after venepuncture, the heart rate decreased in adults by 6–57 bpm, while in juveniles a slight increase of this parameter was observed. In fact, 15 animals (50% of the sample) showed an increase in heart rate during the venepuncture procedure. Of these, 40% were adults and 60% juveniles.

The same was not observed for respiratory rate and rectal temperature. Respiratory rate remained relatively stable throughout the whole procedure although there was a decrease on the mean values obtained at the beginning and at the end of the method of 7 bpm ($p = 0.0066$). Neither sedation nor venepuncture caused statistically significant changes to the baseline respiratory rate. The variation registered upon sedation ranged between a reduction of 32 bpm to an increase of 24 bpm. Increase in the respiratory rate was observed in 10 animals (30% adults and 70% juveniles). Regarding the venepuncture, variation in the respiratory rate ranged between a reduction of 40 bpm to an increase of 12 bpm. Increase in the respiratory rate was only observed in seven animals.

Rectal temperature decreased gradually throughout the whole procedure ($p < 0.05$), with a mean difference between BS and BC of -0.8 ± 0.9 and between BC and AC of -0.6 ± 0.7°C.

Considering all the procedure, minimum and maximum values observed for heart rate (beats per minute) were 72 and 207, for respiratory rate (breaths per minute) 28 and 88 and for temperature (Celsius grades) 34.3°C and 40.2°C, respectively. All these changes are shown in Table 4 and Fig. 3.

Neither morbidity nor mortality were observed by the persons who monitor the areas where the animals were captured and released, including 14 animals that were camera monitored for five days (Fig. 4). Calcium oxide labeling revealed no discomfort from the animals during application

Table 2. Morphological parameters of the adult and juvenile rabbits used in this study. The values presented are the mean $\pm$ SD. Same letters and different letters indicate, respectively, respectively indicate the absence and existence of a statistically significant differences.

Morphological parameters	Juveniles (n = 14)	Adults (n = 16)	p-value	
Weight	568 ± 128[a]	956 ± 124[b]	0.000	g
Body length	22.6 ± 1.9[a]	26.3 ± 2.5[b]	0.000	cm
Head length	7.3 ± 0.8[a]	8.1 ± 0.6[b]	0.002	
Ear length	7.5 ± 0.5[a]	7.8 ± 0.6[a]	0.064	
Tarsus length	3.9 ± 0.3[a]	4.5 ± 0.6[b]	0.001	

Table 3. Comparison of the clinical parameters according to the capture method used.

Clinical parameters		Mean ± SD
Ferrets (n = 5)	heart rate	201 ± 97
	respiratory rate	51 ± 23
	rectal temperature	37.7 ± 3.4
Trap (n = 25)	heart rate	164 ± 46
	respiratory rate	58 ± 14
	rectal temperature	38.2 ± 2.7

and on the following days and allowed rapid and clear identification in the chambers either day or night.

Genetic analysis

Genetic analysis confirmed that all tested rabbits (n = 12, 40% of total sampling), belong to the *O. c. algirus* subspecies (results not shown).

Discussion

The technique described for EJV blood collection in apparently healthy wild rabbits *Oryctolagus cuniculus algirus* under sedation with midazolam has proven to be safe and effective for sampling relatively high volumes of blood (1 ml) in this wild species.

To correctly execute the procedure, the technician must be aware of the morphological and physiological particularities of this fragile species to minimize the associated risks.

During the procedure, keeping stress to a minimum has proven crucial for sedation effectiveness and successful venepuncture at first attempt while it is also important to prevent heart ischemia induced by coronary vasoconstriction since rabbits have limited collateral coronary circulation (Quesenberry and Carpenter 2012).

Also, care was taken to avoid injuring, particularly bone fractures. Vertebral fracture, usually observed at the seventh lumbar vertebra with spinal cord damage, can occur if the animals are not held securely when picked up (Brewer and Cruise 1994). More stressed rabbits were carried with their head under the handler's arm to minimize stress by covering the eyes to limit vision, as recommended in literature (Graham 2006). At the time of the sedative administration, there were no signs of pain in the animals. After the onset of sedation, the animals were handled much more easily, as hind limb movements and attempts to escape were drastically reduced.

As wild rabbits' veins are extremely thin and the most peripheral ones susceptible to hematoma formation (Quesenberry and Carpenter 2012), after venepuncture and needle withdrawal, enough pressure was applied to limit hematomas. Throughout the procedure, both the handler and bleeder constantly monitored the animals' breathing pattern and the colour of the oral mucosa to guarantee proper oxygenation.

Blood collection from the EJV can stimulate the vagus nerve, resulting in heart arrhythmias that may range in severity from bradycardia to complete sinoatrial or atrioventricular block (Hegedus and Shackelford 1965). None of these events were observed during the procedures. This was confirmed by constant clinical observation of the animal and continuous electrocardiogram monitoring. To avoid vagus nerve stimulation and carotid artery laceration, the bleeder ensured that the attempts to locate the vessel, once the needle passed the skin, were done superficially, as recommended by Nelson et al. (2010).

Data obtained from our work showed that heart rates taken prior to sedation ranged between 117 and 292 bpm. There is no reference range for the wild rabbit physiological parameters in the literature. Considering the interval values described for the European rabbit (O'Malley 2005) of 180–250 bpm as a reference, some of our values detected before sedation are relatively low. This can be explained by the fear bradycardia mechanism explained below.

The large interval of heart rate observed in the animals can be explained by differences among the animals, such as environmental responses, sensibility to midazolam, initial physiologic conditions, social status, alongside others (Eisermann 1988). However, in four animals (13.3%), the heart rate increased 4–32 bpm after sedation. This can either be a result of individual higher sensibility and susceptibility to midazolam effects or higher stress due to conservation of the animals in the black bag during the sedation onset. The time considered as onset of sedation was somehow subjective, since it was based on the animal's response to restraint and general behaviour. Consequently, measurement of each physiological parameter may have taken place at different depths of sedation.

The mean increase of heart rate that we observed in juveniles can be explained by 1) different metabolic rate that can influence the pharmacokinetics of midazolam, 2) major stress that these animals are prone to at this age class, 3) individual cardiorespiratory response to the effect of benzodiazepines. Furthermore, the continued reduction in heart rate observed in the other 15 animals may also be explained by a more prolonged sedation in these rabbits.

Table 4. Physiological parameters collected from rabbits submitted to EJV venepuncture. The values of heart rate and respiratory rate are per minute and the value of rectal temperature in °C. Same letters and different letters indicate, respectively, respectively indicate the absence and existence of a statistically significant difference.

Clinical parameters	BS	BC	AC	p-value
Heart rate (mean ± SD)	164 ± 39[a]	119 ± 27[b]	121.7 ± 29.5[b]	<0.0001 BC–AC = 0.6
Respiratory rate (mean ± SD)	58 ± 13[a]	56 ± 15[ab]	51.0 ± 10.3[b]	<0.0001 BS–BC = 0.5 BC–AC = 0.1
Rectal temperature (mean ± SD)	38.2 ± 1.2[a]	37.4 ± 1.1[b]	36.8 ± 1.2[c]	<0.0001

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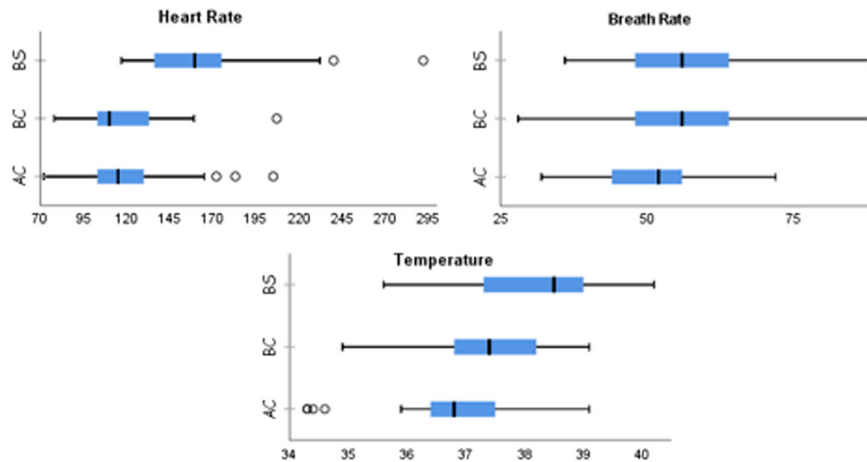
Figure 3. Boxplot graphs depicting heart rate (beats per minute on x-axis), respiratory rate (breaths per minute on x-axis) and temperature (°C on x-axis) evolution in three moments for each graph (before sedation-top; after sedation and before sampling – midline; after sedation and after sampling-down). X represents mean value. The three graphs only represent different viewing angles.

Besides heart rate variation due to sedation, fear bradycardia was also observed in some animals during manipulation. In consequence of the high levels of predation that wild rabbits are subject to, their sensory organs are highly developed. Rabbits are sensitive to catecholamines and have evolved for flight rather than fight, meaning they respond to threat passively by crouching and hiding (Smith et al. 1981). Animals that freeze in situ or drop into a burrow often show a sudden and marked fear bradycardia (Smith et al. 1981), a poorly understood, widely observed, alternate fear response. In lagomorphs, fear bradycardia appears to be due to a reduction of sympathetic activity and an increased parasympathetic activity (Smith et al. 1981). Since it occurs in atropine treated animals, it has been suggested that this type of response is, at least, partly mediated by removal of sympathetic tone (Smith and Worth 1980). Causby and Smith (1981) described a decrease in heart rate of 53% at −11.2 bpm s⁻¹ in *Sylvilagus aquaticus*. Keeping our animals in boxes and bags, making

them unable to flee, probably led to the onset of this mechanism during our study.

The rectal temperature decreased since the beginning of the procedure. The reduction of this physiological parameter may have resulted from 1) a decrease in the metabolism of the animals resulting in a reduction of heat production, 2) a reduction in the muscular movements due to containment of the animal, resulting also in a lower heat production and 3) the increment of heat loss due to use of ethanol to wet the animal's fur, due to evaporation. In this context, ethanol can be replaced by another disinfectant with lower heat evaporation effects such as chlorhexidine.

The midazolam dose (1 mg kg⁻¹) used in this study was considered sufficient for the short duration of the procedure. In field conditions, without resuscitation equipment and other supporting means available, it is critical to ensure that the technique in use has negligible risks associated. When using this low dose, individual variation has a greater impact on the depth of sedation, with the animals exhibiting different responses. However, given that the safety margin between anaesthetic and lethal dose in the rabbit is narrower than in other species, and that individual variation is larger (de Vries et al. 2007), the use of this dose (1 mg kg⁻¹ midazolam) is recommended.

Despite rabbits' susceptibility to overheating (Wildpro 2017), during our study a steady decrease of body temperature throughout the whole procedure was observed, possibly because most of the blood collections were made during winter (with temperatures ranging between 7°C and 20°C).

From our experience, restraint of the animal by an experienced person and correct identification and visualization of the EJV proved critical to guarantee the success of the procedure, in the first attempt. Presenting the animal correctly to the person in charge of the venepuncture was essential for visualization and less deviation of the EJV, located laterally to the midline and overlying the internal jugular vein and the carotid artery. Shaving the neck over the midtrachea cranial to the thoracic inlet (Quesenberry and Carpenter 2012) can be carried out for a better EJV visualization. If shaving must be performed, electric hair clippers with thin blades

Figure 4. Photographs taken after release of the rabbits. Left- at night (arrow indicates the marking on the ear); right-during the day (arrow indicates the marking on the ear).

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(size 50, 1/1 in. [0.2 mm], or 40, 1/100 in. [0.25 mm]) (Quesenberry and Carpenter 2012) must be used, because rabbit fur is very thin, and the animal's coat must be clean and dry.

The technician must also be aware that clipping off large amounts of fur or using copious quantities of disinfectant during skin preparation potentiate heat loss and the development of hypothermia, especially in small rabbits with no fat reserves (Varga 2014). Whenever the procedure is to be performed in a timely manner, and once the person performing the procedure has acquired experience, this step can be avoided to minimize stressing the animal and prevent skin exposure to hives and other aggressive plants.

For most cases, where only 0.5 ml of serum was necessary, 1 ml syringes were used given the low negative pressure on the vein. However, in adult animals, or whenever required, a 2 ml syringe can also be used. If this is the case, to minimize the vacuum exerted on the wall of the EJV, the vacuum was withdrawn from the syringe before the animal was punctured.

Whenever the first attempt to collect blood failed, the needle should be slowly removed, and the puncture site monitored for bleeding. More than two attempts should be avoided in case of bleeding due to the risk of vein collapse.

Undoubtedly, in studies involving live animals it is most important to safeguard the individual variability among the specimens. The use of wild specimens from different geographical areas, captured by different methods, with different levels of habituation to humans, leads to a multiplicity of factors that may influence the animals' physiological parameters. In addition, the unknown sanitary condition of the animals due to parasitism, or to any underlying subclinical infection, may also influence the physiological conditions of the animals.

The studies of pathologies in the species are reliant upon dead animals or stool samples found in the fields. This study represents a breakthrough in this area, by allowing in vivo biological sampling of a wild species. Access to blood sampling creates the opportunity for year-round generalised surveys, as well as the evaluation of physiological parameters (e.g. blood count and biochemistry analysis) of this species, without depending on cadavers either obtained during the hunting season or found dead.

Moreover, release of the sampled specimens is critical to avoid disturbance of recovery in diminished populations. The EJV technique allowed the collection of 1mL of blood from both adults and juveniles. However, researchers should adhere to institution specific guidelines regarding permitted maximum volumes and frequency of blood collection. There was neither significant bruising nor any other injuries before, during or after the venepuncture procedure and sedation. Additionally, there were also no injuries inflicted on the manipulators.

Complementary procedures to assess the influence of the sedation and venipuncture on the physiology of the animal by temperature assessment and monitoring of heart and respiratory rates, were also described in this manuscript to validate the method. When complementary monitoring is no further necessary, the time needed for sedation and blood collection is drastically shortened, as well as the stress level.

The method can potentially be adapted to other species, especially to other small mammals, allowing longitudinal studies and supporting other conservation methods.

References

Abade dos Santos, F. A. et al. 2017. Detection of rabbit Haemorrhagic disease virus 2 during the wild rabbit (*Oryctolagus cuniculus*) eradication from the Berlengas Archipelago, Portugal. – BMC Vet. Res. 13: 336.

Abrantes, J. et al. 2013. New variant of rabbit hemorrhagic disease virus, Portugal, 2012–2013. – Emerg. Infect. Dis. 19: 1900–1902.

Angulo, E. 2004. El Conejo, el monte mediterraneo en Andalucía. – Cons. Medio Ambient. Junta Andalucia, Sevilla.

Bell, D.J. and Webb, N.J. 1991. Effects of climate on reproduction in the European wild rabbit (*Oryctolagus cuniculus*). – J. Zoology 224: 639–648.

Borkowski, R. and Karas, A. Z. 1999. Sedation and anesthesia of pet rabbits. – Clin. Tech. Small Anim. Pract. 14: 44–49.

Boyle, J. E. 2016. Crow and Walshaw's manual of clinical procedures in dogs, cats, rabbits and rodents, 4th edn. – Wiley.

Brewer, N. and Cruise, L. 1994. Physiology. – In: Manning, P. et al. (eds), The biology of the laboratory rabbit, 2nd edn. Academic Press, pp. 63–70.

Buil, T. et al. 2004. Critical points in the transport of commercial rabbits to slaughter in Spain that could compromise animals' welfare. – World Rabbit Sci. 12: 269–279.

Cantwell, S. L. 2001. Ferret, rabbit and rodent anesthesia. – Vet. Clin. North Am. Exot. Anim. Pract. 4: 169–191.

Carvalho, Silva, S. et al. 2017. Emergence of rabbit haemorrhagic disease virus 2 in the archipelago of Madeira, Portugal (2016–2017). – Virus Genes 53: 922–926.

Causby, L. A. and Smith, E. N. 1981. Control of fear bradycardia in the swamp rabbit, *Sylvilagus aquaticus*. – Comp. Biochem. Physiol. C Comp. Pharmacol. 69: 367–370.

Cizek, L. J. 1961. Relationship between food and water ingestion in the rabbit. – Am. J. Physiol. 201: 557–566.

de Vries, H. W. et al. 2007. Four methods for general anaesthesia in the rabbit: a comparative study. – Lab. Anim. 22: 355–360.

Delibes, M. et al. 2000. Action plan for the conservation of the Iberian lynx (*Lynx pardinus*) in Europe. – Counc. Eur. Publ. Nat. Environ. Strasbourg, Fr. 111.

Duarte, M. et al. 2015. Rabbit haemorrhagic disease virus 2 (RHDV2) outbreak in Azores: disclosure of common genetic markers and phylogenetic segregation within the European strains. – Infect. Genet. Evol. 35: 163–171.

Eisermann, K. 1988. Seasonal and environmental influences upon the diurnal heart-rate pattern in wild rabbits living under seminatural conditions. – Physiol. Behav. 43: 559–565.

Flecknell, P. A. 1984. The relief of pain in laboratory animals. – Lab. Anim. 18: 147–160.

Flecknell, P. A. 1987. Laboratory animal anaesthesia. – Academic Press.

Gálvez-Bravo, L. 2017. Conejo – *Oryctolagus cuniculus*. Salvador, A. and Barja, I. (eds), Enciclopedia virtual los vertebrates. Mus. Nac. Ciencias Nat. Madrid. <www.vertebradosibericos.org/>.

Gargiulo, S. et al. 2012. Mice anesthesia. – ILAR J. 53: E55–E68.

Gibb, J. A. 1990. The European rabbit. – In: Chapman, J. A. and Flux, J. E. (eds), Rabbits, hares and pikas: status survey and conservation action plan. IUCN, Switzerland, 116–120.

Gibb, J. A. and Williams, J. M. 1990. European rabbit. – In: King, C. M. (ed.), Handbook of New Zealand mammals. Oxford Univ. Press, pp.138–160.

Graham, J. 2006. Common procedures in rabbits. – Vet. Clin. North Am. Exot. Anim. Pract. 9: 367–388.

Hegedus, S. A. and Shackelford, R. T. 1965. A comparative–anatomical study of the craniocervical venous systems in mammals, with special reference to the dog: relationship of anatomy to measurements of cerebral blood flow. – Am. J. Anat. 116: 375–386.

Henke, J. et al. 2005. Comparative study of three intramuscular anaesthetic combinations (medetomidine/ketamine, medetomidine/fentanyl/midazolam and xylazine/ketamine) in rabbits. – Vet. Anaesth. Analg. 32: 261–270.

La Fuente, F. R. 1993. Cadernos de campo. – A Lebre e o Coelho, Lisboa.

Longley, L. et al. M. 2008. Anaesthesia of exotic pets. – Elsevier.

Macdonald, D. W. and Barrett, P. 1993. Mammals of Europe. – Prineton Univ. Press.

Muir, W. W. et al. 2000. Drugs used for preanesthetic medication. – Handb. Vet. Anesth. 3: 19–40.

Mullan, S. and Saunders, R. 2018. European rabbits (*Oryctolagus cuniculus*). – Companion Anim. Care Welf. 163–184.

Nelson, E. A. et al. 2010. A jugular bleeding technique in rabbits. – Lab. Anim. 39: 17–22.

O'Malley, B. 2005. Clinical anatomy and physiology of exotic species. Structure and function of mammals, birds, reptiles and amphibians, 1st edn. – Elsevier.

Parasuraman, S. et al. 2010. Blood sample collection in small laboratory animals. – J. Pharmacol. Pharmacother. 1: 87.

Patrick, J. et al. 1994. The biology of the laboratory rabbit, 2nd edn. – Elsevier.

Quesenberry, K. E. and Carpenter, J. W. (eds) 2012. Ferrets, rabbits and rodents clinical medicine and surgery. – In: Clinical medicine and surgery. 3rd edn. ScienceDirect.

Ramer, J. C. et al. 1999. Evaluating and stabilizing critically ill rabbits. I. – Compend. Contin. Educ. Pract. Vet.

Seamer, J. 1993. Farm animal welfare in Britain. – SCAW (Scientists Cent. Anim. Welfare) Newsl. 14: 13–14.

Smith, E. N. et al. 1981. Fear bradycardia in captive eastern chipmunk, *Tamias striatus*. – Comp. Biochem. Physiol. Part A Physiol. 70: 529–532.

Smith, E. N. and Worth, D. 1980. Atropine effect on fear bradycardia of the eastern cottontail rabbit, *Sylvilagus floridanus*. – In: Amlaner, C. J. and Macdonald, D. W. (eds), Handbook on biotelemetry and radio tracking. ScienceDirect, pp. 549–555.

Varga, M. 2014. Textbook of rabbit medicine. – Elsevier.

Virgós, E. et al. 2005. El declive del conejo en España: evidencias a partir de las estadísticas de caza. – Quercus 236: 16–20.

Von Holst, D. et al. 1999. Social rank, stress, fitness, and life expectancy in wild rabbits. – Naturwissenschaften 86: 388–393.

Ward, D. 2005. Reversing rabbit decline: one of the biggest challenges for nature conservation in Spain and Portugal. – SOSLynx.opg.

Wildpro 2017. Detailed physiology notes with literature reports for the European rabbit – *Oryctolagus cuniculus*. – <http://wildpro.twycrosszoo.org/S/0MLagomorph/Leporidae/Oryctolagus/Oryctolagus_cuniculus/10Oryctolagus_cuniculus DetPhy.htm, accessed 14 November 2017.

Willott, S. J. et al. 2000. The contribution of rabbits (*Oryctolagus cuniculus* L.) to soil fertility in semi-arid Spain. – Biol. Fertil. Soils 31: 379–384.

Are hare primary cells suitable for isolation of new viruses?

Scientific publication 2

Simple Method for Establishing Primary Leporidae Skin Fibroblast Cultures

■ ■

Primary Leporidae skin fibroblast cultures were established to be used in the leporid herpesvirus, among other viruses, study and isolation.

Protocol

Simple Method for Establishing Primary Leporidae Skin Fibroblast Cultures

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Abstract: Commercial hare and rabbit immortalized cell lines are extremely limited regarding the many species within the lagomorpha order. To overcome this limitation, researchers and technicians must establish primary cell cultures derived from biopsies or embryos. Among all cell types, fibroblasts are plastic and resilient cells, highly convenient for clinical and fundamental research but also for diagnosis, particularly for viral isolation. Here, we describe a fast and cheap method to produce primary fibroblast cell cultures from leporid species, using dispase II, a protease that allows dermal–epidermal separation, followed by a simple enzymatic digestion with trypsin. This method allows for the establishment of an in vitro cell culture system with an excellent viability yield and purity level higher than 85% and enables the maintenance and even immortalization of leporid fibroblastic cells derived from tissues already differentiated.

Keywords: primary fibroblasts; primary cell culture; virus isolation; method; dispase II; Leporidae

1. Introduction

Leporids comprise different species of hares and rabbits that have drawn the attention of the scientific community (e.g., as human diseases models, to produce immune sera and monoclonal antibodies, for example), the farming sector, the general public, and conservationists for very different reasons. These range, from its use as models for human diseases, livestock for human consumption, pets, game species, and for their notorious role in the preservation of important ecosystems and biodiversity. Since leporid pathogens can impact a wide range of areas and sectors, the study of their diseases is of paramount importance. Viral isolation is a basic tool in the diagnosis of well-described and unknown viruses, as well as for fundamental research that enables the understanding of the pathogenesis of several diseases through the study of every step that defines the virus–host cell interactions. Moreover, these interactions require in vitro cultured cells permissive to the infection, which allows the binding of viral capsid (for naked viruses) or viral envelope glycoproteins to the receptors present at the cell membrane, which are specific to each cell phenotype.

Primary cells are obtained regularly from living tissues, most often from embryonic sources, and are subsequently submitted to in vitro culture procedures. Despite some disadvantages when compared to immortalized cell lines, namely the inability to survive after a limited number of in vitro passages [1], primary cells preserve the same characteristics as those from the originating tissue, facilitating studies in many research areas, namely in

virology and cell biology that otherwise would be far more complicated. Additionally, they are relatively cheap and easy to obtain and may be prepared from any animal species.

The hare and rabbit immortalized cell lines currently available are limited to epithelial-like kidney cells (RK13 from rabbit and HN-R and a few more from European hare), and to skin primary fibroblasts from New Zealand rabbits that are also commercially available. However, the specificity of certain studies requires the preparation of primary cell cultures from the host under analysis. This is the case, for instance, for skin fibroblasts useful for investigating Iberian hare (*Lepus granatensis*) viruses.

Fibroblasts are mesenchymal-derived cell types, involved in the synthesis of extracellular matrixes (ECMs), such as collagen, elastin, fibronectin, and others, and the secretion of growth factors. They play a central role in maintaining structural homeostasis as water-holding glycoproteins [2].

Fibroblasts are frequently used to study cellular biological processes, for clinical research, toxicological studies, microbiological investigations, among others. In the virology field, cultured fibroblasts are very useful for primary isolation and the adaptation of wild type virus strains since the number of viral particles in infected tissue samples obtained from primary or secondary replication organs in field isolates is usually low. Furthermore, viruses do not grow in cells derived from heterologous species, even when expressing the appropriate receptor [3,4]. Fibroblasts cell cultures are one of the easiest to be established, as no complex purification processes are required and, depending on the species of origin, cells can even be prepared from samples collected from live animals without need of anesthesia, sedation, or sacrifice. This is the case for Leporidae. There are many protocols available with different levels of complexity, approaches, and objectives, some of which are summarized in Table 1. The protocol presented here is the first optimized for obtaining primary fibroblasts from live wild leporids. The method is simple, low cost and enables large amounts of cells to be obtained and, therefore, worthy of sharing. The protocol uses dispase II, a proteolytic enzyme, followed by a simple incubation with trypsine solution. Despite the method having been implemented and optimized for Iberian hare (*Lepus granatensis*) and wild rabbit (*Oryctolagus cuniculus algirus*) fibroblast cultures, it can be applied to biological samples of any species, proving to be easy and quick to perform.

Table 1. Available techniques for primary fibroblasts isolation.

Species Used	Tissue Type	Harvest	Technique	Notes	Reference
Human	Skin biopsies from anterior surface of the forearm	HAM-F10 cell culture medium with 20% FBS	Adhesion of fragments to the flask surface	Large maintenance costs of culture medium during initial fibroblast growth.	[5]
Human	Skin biopsy	DMEM cell culture medium with 20% FBS	Adhesion of fragments to the well bottom	Estimation of 25–35 days to second passage. Large maintenance costs of culture medium during initial fibroblast growth	[6]
Human	Skin biopsy	Complete DMEM or complete RPMI	Primary explant method without/ with epidermis removal with 0.5% dispase/ PBS or 0.3% trypsin/PBS	Confluence is generally reached in ∼3 to 5 weeks.	[7]
Mouse	Skin from tail and ear	RPMI: 10% fetal calf serum (FCS), 50 µM 2-mercaptoethanol, 100 µM asparagine, 2 mM glutamine, 1% penicillin-streptomycin solution.	Digestion with collagenase and D-pronase solution	Euthanasia was used. The fragments correspond to epidermis and dermis to ensure the grow of keratinocytes is probable.	[8]

2. Material and Methods

2.1. Tissue Sample Collection

All biological samples used in this study were collected from wild Leporidae in captive centers by skin punch biopsy for genetic analysis or during animal marking. The tool used is commercially available (Figure 1A). This study was conducted in the scope of a project called +Coelho that operationalizes the Action Plan for the Control of Rabbit Hemorrhagic Viral disease in Rabbits (Dispatch 4757/17 of 31 May), specifically approved by the Portuguese National Authority for Animal Health (Authorization 79/ECVPT/20145). No animals were subjected to housing or handling for the exclusive purpose of this study. All sampling procedures were performed by veterinarians.
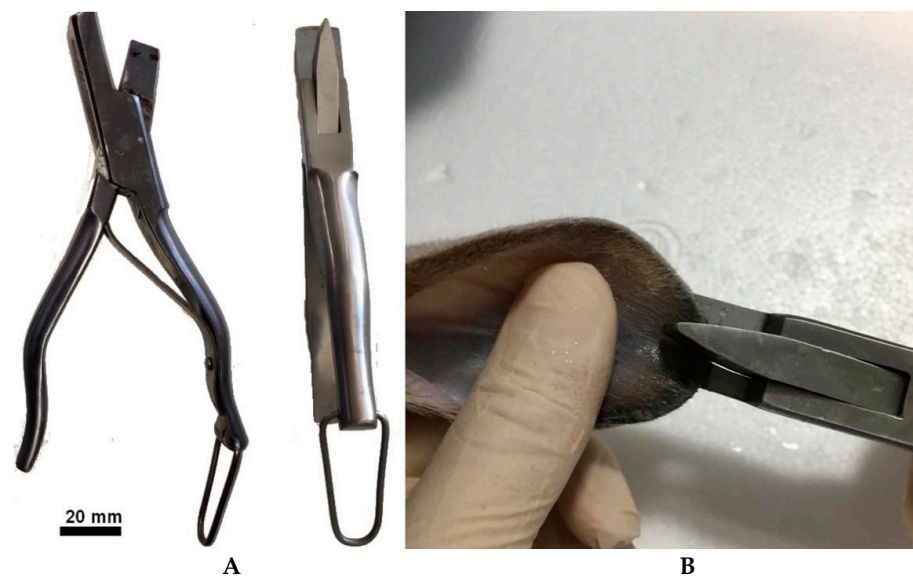
Figure 1. (**A**) Commercial equipment used for skin sample. (**B**) Collection of an ear sample.

After collection in the field or in the animal premises, the tissue samples were stored in culture medium (DMEM/HAMS F12 medium with L-Glutamine and HEPES (3.5 g/L) (CORNING), supplemented with 200 units/mL of penicillin, 200 µg/mL of streptomycin, 0.5 µg/mL of Gibco Amphotericin B, and 50 µg/mL of gentamicin) at room temperature (RT) for a maximum of two hours, and then incubated at 37 °C for a maximum of three hours prior to preparation. For longer storages, 50-milliliter Falcon tubes or T25 culture flasks are recommended. During storage at 37 °C, the medium should be replaced every hour.

Ideally, skin samples should be obtained from healthy juvenile animals, rather than adults. However, this protocol was successfully used with adult skin samples.

Before harvesting, asepsis of the biopsy ear area was carried out by seven washes with a chlorhexidine solution using swabs, followed by one wash with sterile 0.9% NaCl. The biopsy of the ear was performed using a biopsy punch (Figure 1B). Samples were taken from the margin of the ear, avoiding major blood vessels that can easily be visualized against light. If correctly performed, no hemorrhage, pain, inflammation, or infection is produced during and after biopsy.

In docile animals (e.g., domestic rabbits) the area can be clipped, but this procedure should be avoided in wild animals sensitive to clipping noise and vibration.

2.2. Reagents

For this protocol only two reagents are required, namely dispase II (42613-33-2, Sigma-Aldrich, St. Louis, MO, USA) and a trypsin dissociation solution (0.8% NaCl, 5 mM KCl, 5.5 mM Glucose-Dextrose, 7 mM Na_2HPO_4, 1.25 g of Trypsin 1:250, Sigma-Aldrich, pH 7.2 $\pm$ 0.2, filtered using a 0.22-micrometer mesh).

2.3. Preparation of Fibroblasts from Skin Biopsy

All steps should be performed in a laminar flow hood using sterile using sterile disposable material.

(1) Wash the original tissue fragments to remove impurities by placing the sample in a 50-milliliter Falcon tube containing 30 mL DMEM/HAMS F12 medium with L-Glutamine and HEPES (3.5 g/L) (CORNING), 5× Antibiotic–Antimicotic solution (200 units/mL of penicillin, 200 µg/mL of streptomycin, 0.5 µg/mL of Gibco amphotericin B and 50 µg/mL of gentamicin); immediately proceed with vigorous washing, using a shaking robot or just manual shaking. Change medium seven times after 1-min-long agitation.

(2) Place the original tissue fragments in 50-milliliter Falcon tubes (or alternatively in T25 bottles, or Petri dishes) and fill with DMEM medium prepared as described below.

(3) Remove the medium and add a volume of 0.2 mg/mL dispase II prepared in DMEM medium enough to cover the tissue fragments. The epidermis–dermis separation process takes a variable time depending on the dimension, shape, and thickness of the fragments, generally 1–2 h at 37 °C. We recommend shortening this procedure as much as possible, since hair and epidermis are contaminated by bacteria, fungi, and yeasts, even after washing, and must be removed from the preparation as soon as possible. The operator must, therefore, test the detachment every hour, although cells may be left overnight in this medium with no adverse effects. Remove the epidermis completely using two tweezers (Figure 2B,C) and wash the epidermis fragment with DMEM medium as often as necessary until no hairs are visible (Figure 2C).

(4) Cut the original fragments into smaller fragments less than 5 mm wide, using a scalpel (Figure 2D).

(5) Incubate the fragments with the trypsin dissociation solution (the smallest volume that covers the fragments), previously warmed to 37 °C, in 50-milliliter tubes, T25 culture flasks or Petri dishes, and incubate at 37 °C for 10 min. The dissociation solution becomes cloudy as cells detach. Caution must be taken at this step since trypsin activity continues beyond 30 min, destroying the cells.

(6) Remove the enzymatic digestion solution containing the cells and centrifuge at 150× *g* for 10 min. Resuspend the cells in a conical tube containing DMEM/HAMS F12 medium with L-Glutamine AND HEPES (3.5 g/L), 5× antibiotic-antimycotic solution (100 units/mL of penicillin, 100 µg/mL of streptomycin, 0.5 µg/mL of Gibco amphotericin B, and 25 µg/mL of gentamicin) and 10% of fetal bovine serum (Sigma-Aldrich, St. Louis, MO, USA).

(7) Add fresh dissociation solution to the tissue fragments and repeat steps (5) and (6) as many times as necessary.

(8) Seed the cells in culture flasks. Incubate at 37 °C with 5% CO_2.

(9) Remove medium after 2–4 h incubation, wash the cells with DMEM, and add fresh medium.

(10) Remove medium again after 2–4 h of incubation. Wash the cell layer with DMEM and add fresh medium.

The presence of dead cells in suspension during the first 48 h of incubation is expected. Due to the normal skin microbiome, some cultures may show signs of contamination after 24–48 h incubation and, therefore, must be eliminated. In plastic surfaces where the initial cell density is low, supplementation of the culture media with 20% fetal bovine serum is recommended in order to assure cell viability and turnover of mitosis. The percentage of fibroblasts in culture using this technique exceeds 85% (evaluated using morphological analysis).

Plated cells (Figure 3A,B) assume the typical spindle-shaped morphology and reach 60 to 70% confluence between days 2 and 3 of incubation. Although the purity of these cultures in fibroblasts reaches proportions of 75% or higher, morphologically distinct cells, namely epithelioid or stellate-like cells, are observed in the cultures using optical-phase microscopy. The different morphologies assumed by the fibroblasts are shown in Figure 3C,D.
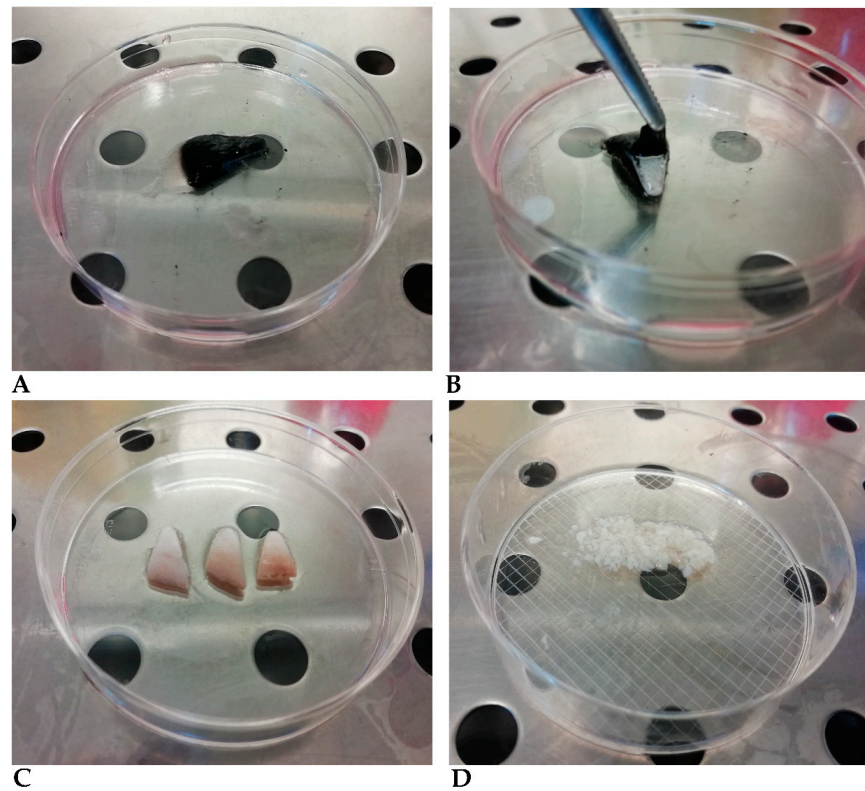
Figure 2. (**A**) Ear sample before dispase II treatment, (**B**) Ear sample after incubation with dispase II, showing the detach of the epidermis, (**C**) Ear sample after epidermis removal, (**D**) Dermis fragments cut to less than 5 mm diameter dimensions.
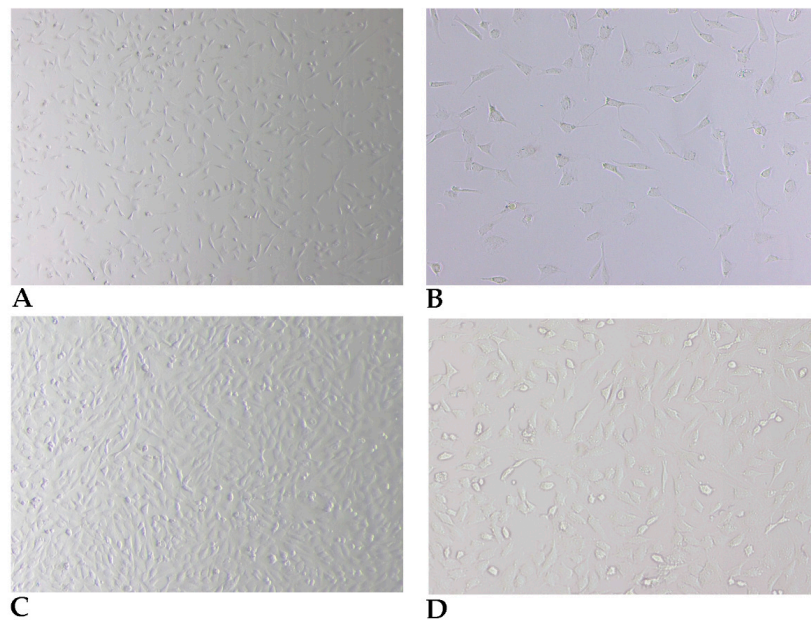
Figure 3. Phase contrast microscopy of typical fibroblast cells after step (7) of the *Preparation of fibroblasts from skin biopsy* procedure, at day 2, 40× (**A**) and 100× (**B**), and at day 4, 40× (**C**) and 100× (**D**).

2.4. Subculturing and Harvesting Primary Fibroblast Cells

Fibroblast cells have a high multiplication ratio and must be subcultured when the cell layer reaches about 90% confluence.

(1) Remove the medium and wash cells twice with sterile PBS.
(2) Incubate cells with trypsin-EDTA (0.25%), enough to cover the cell layer, and incubate at 37 °C until cell detachment.
(3) Centrifuge the cell suspension at $150 \times g$ for 10 min and recover the pellet.
(4) Cells are counted in a Neubauer hematocytometer and plated at $1–2 \times 10^4$ cells/cm^2

Note: Fibroblasts are not contact inhibited. The primary fibroblasts are particularly sensible to the trypsinization (comparing with typical immortalized cell lines); therefore, their viability is generally lower.

2.5. Viability Assay Using Trypan Blue Dye

Viability of cells after cryopreservation was evaluated by cell counting after trypan blue staining and when necessary, after subculturing and freezing process. Cells were diluted at 1:10 in Trypan Blue, counted in a Neubauer chamber, and frozen.

2.6. Freezing and Thawing Fibroblast Cells

Cryogenic preservation can maintain fibroblast cells for very long periods [9]. Subcultures with 3 or 4 passages should be used for cryogenic preservation.

(1) Trypsinise cells with 70–80% confluence, as described above in Section 2.4 (steps (1)–(3)).
(2) Centrifuge at $150 \times g$ for 10 min and resuspend the cell pellet with 10% DMEM, 10% DMSO, and 80% FBS (*v/v*).
(3) Aliquot the cell suspension in cryogenic storage tubes at a density of $1–3 \times 10^6$ cells/ vial. We have obtained an average of two vials for every three T25 flasks with 90% confluence.
(4) Freeze cells at -80 °C overnight and then transfer them into liquid nitrogen.

To resuscitate cells after liquid nitrogen freezing, vials must be quickly thawed at 37 °C and promptly transferred to cell culture flasks with DMEM medium. After 12–24 h of incubation, wash cells, and add fresh medium.

If strict asepsis techniques are used, it is possible, and recommended, to maintain these cultures without the addition of antibiotics.

2.7. Fibroblast Marker by Immunocytochemistry

In order to check the purity of the culture obtained and confirm the evidence given by the morphological data, immunocytochemistry was performed (Figure 4) using cells grown in 8-well slides (Thermo Scientific™ Nunc™ Lab-Tek™ Chamber Slide System). The first, second and third passages were cultivated and evaluated with 8 replicates for each species (rabbit and hare). Two primary antibodies were used, a monoclonal antibody anti-Vimentin, Clone V9 (Figure 4A,C) that marks fibroblasts, endothelial cells, lymphoid tissue and melanocytes and a monoclonal antibody anti-Cytokeratin AE1/AE3 as epithelial cells marker (Figure 4B,D).

(1) Grow cells in chamber slide system (e.g., (Thermo Scientific™ Nunc™ Lab-Tek™ Chamber Slide System)) until 95% cell confluence is reached.
(2) Remove the medium and wash 2 times with PBS.
(3) Fix cells by adding 100 µL of 100% acetone (we used Acetone, for GC residue analysis (Scharlab, Barcelona, Spain)) to each well and incubate for 10 min. Remove the acetone.
(4) Remove the slides from the chamber slide system. Rehydrate the cells in Dako EnVision FLEX Wash Buffer (Agilent, Santa Clara, CA, USA) with 1% of Triton X-100 for 5 min.
(5) Wash the cells with Dako EnVision FLEX Wash Buffer.
(6) Delimitate the cell wells with a Dako Pen.
(7) Incubate the cells with EnVision FLEX Peroxidase-Blocking Reagent for 10 min.
(8) Wash the cells with Dako EnVision FLEX Wash Buffer.

(9)   Incubate the cells at RT with Monoclonal Mouse Anti-Human Cytokeratin Clones AE1/AE3 (Dako, M3515) and Monoclonal Mouse Anti-Vimentin Clone V9 (Dako, M0725), both diluted 1:100 in EnVision FLEX Antibody Diluent.

(10) Wash the slides with Dako EnVision FLEX Wash Buffer, 2 times for 5 min, with manual shaking. Change the buffer between washes.

(11) Incubate with Envision FLEX/HRP for 30 min (Dako, SM802) at RT.

(12) Wash the slides with Dako EnVision FLEX Wash Buffer, 2 times for 5 min, with manual shaking. Change the buffer between washes.

(13) Incubate with Envision FLEX DAB and Chromogen dilution in the respective buffer, for 3–5 min at RT.

(14) Wash in distilled water.

(15) Nuclei counterstaining with hematoxylin solution for 5 min at RT.

(16) Dehydrate the cells by incubation of ethanol in the following sequence: 70, 80%, 95, and 100%, 1 min each, followed by 2 baths in xylene, 1 min each.

(17) Mount the slides with Slide Mounting Media.
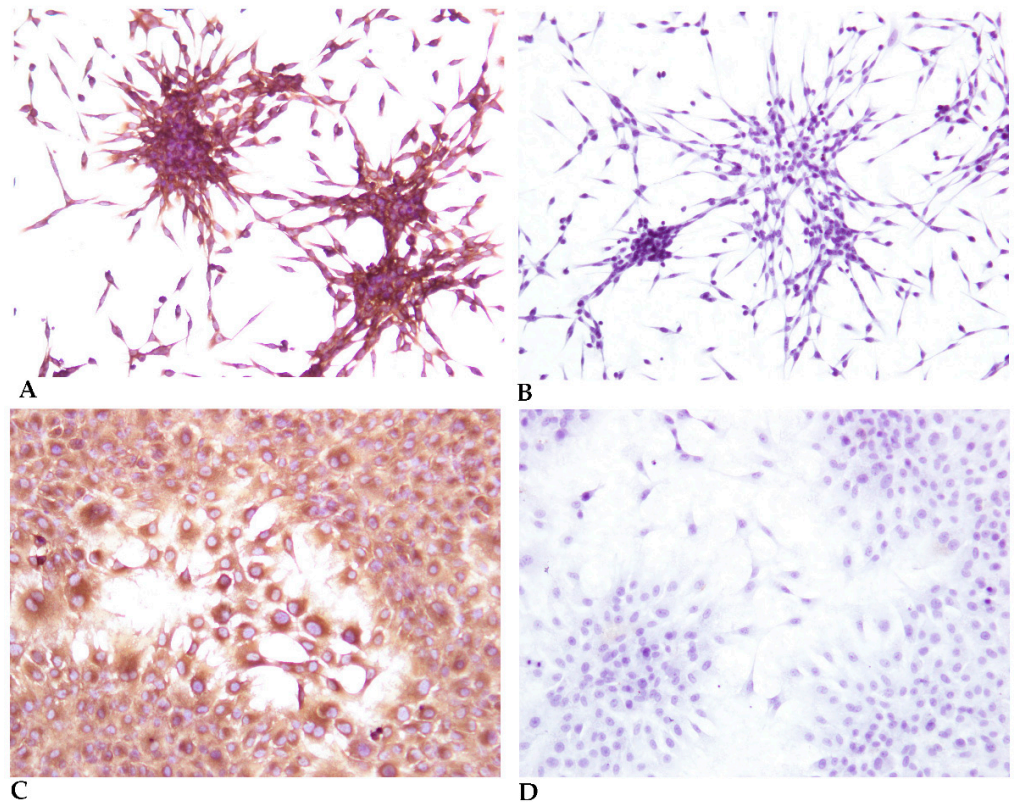
Figure 4. Immunocytochemistry of the third passage (subculture) (**A**) Immunocytochemistry using an anti-Vimentin Clone V9 antibody, of a 20% confluent culture, showing typical spindle cell morphology, 40×; (**B**) Immunocytochemistry using an anti-Cytokeratin AE1/AE3 antibody, of a 20% confluent culture, showing typical spindle cell morphology, 40×. Cells were fixated at lower confluence to allow a better observation of the cell morphology. (**C**) Immunocytochemistry using an anti-Vimentin Clone V9 antibody, of a 70% confluent culture, 100× (**D**) Immunocytochemistry using an anti-Cytokeratin AE1/AE3 antibody, of a 70% confluent culture, 100×.

3. Discussion and Conclusions

Iberian hare and European wild rabbit are the natural hosts and reservoirs of many known multiple species-specific (e.g., myxoma virus, Shope fibroma virus) and zoonotic (e.g., Hepatitis E virus, *Francisella tularensis*) pathogens, and of others yet unidentified that need to be characterized. In this manuscript we describe, for the very first time, a simple protocol for establishing an in vitro model system of fibroblastic cells already differentiated,

derived from biopsies. Moreover, this methodology can be successfully applied to tissue samples derived from healthy adult animals, or even shortly after death (up to two hours).

The isolation of fibroblasts from skin biopsies is a fairly common procedure in humans with a few protocols already available in the literature [6,8,10,11]. However, this approach is new in the field of Veterinary Medicine, since reports of virus isolation or vaccine production, including Influenza [12], Newcastle [13], Rabies [14], Pseudorabies [15], among others, used primary cultures of fibroblasts or epithelial cells, derived from tissue samples obtained during embryogenesis, or at the start of the postnatal period.

The main novelty of the in vitro model system of fibroblastic cells described here is its establishment in a short period and, most of all, the great efficiency regarding cell density, viability, and purity. The trypan blue exclusion test showed a mean cell viability of $95.2 \pm 1.1\%$ during the first four subcultures, decreasing afterwards to $73.4 \pm 7.1\%$ after the seventh subculture. No more than eight subcultures were analyzed for this purpose. The cell proliferation rate using 10 replicates was estimated at 31.8 h during the first four passages. The purity of these cell cultures was also assessed by phenotype specific cell markers, namely vimentin (Figure 4) although morphologically the cells are easily identifiable (especially with low confluence). The absence of labelling by pancytokeratin (Figure 4), excludes the presence of contaminant epithelial cells. In the first passage, a fibroblast purity of $85.3 \pm 9.5\%$ was obtained. In the second and third passages, the purity was $99.3 \pm 0.6\%$, considering eight replicates of each passage for each species. Contamination of the fibroblast cell cultures occurred mainly by keratinocytes and other epithelial cells, probably from hair follicles and other skin adnexal structures.

The viability and cell proliferation rate of fibroblasts were similar to the values reported by other authors [10,16]. In addition, differentiated fibroblastic cells may be subcultured over time with no loss of the morphology or organization of the cell layer. Although there are some primary fibroblast cell lines from domestic rabbits commercially available, the genetic differences between both rabbit species of origin and the rabbit species understudied, and between the wild rabbit and the Iberian hare, compelled us to produce primary fibroblast cell cultures. Fibroblasts are undoubtedly the choice that best allows for the primary isolation of viruses in a homologous system and the increase in viral loads through successive replicative cycles, before adaptation to a permissive cell line, which is easier to manipulate.

In conclusion, the method described in this manuscript is a fast and cheap method to establish primary Leporidae skin fibroblast cultures, providing an alternative technique to obtain highly pure primary fibroblast cultures from rabbit and hare skin explants. This method can also be adapted for the epidermis and can be modified to obtain a pure culture of keratinocytes derived from the epidermis (results not shown).

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Wang, Y.; Chen, S.; Yan, Z.; Pei, M. A prospect of cell immortalization combined with matrix microenvironmental optimization strategy for tissue engineering and regeneration. *Cell Biosci.* **2019**, *9*, 1–21. [CrossRef] [PubMed]
2. Hausmann, C.; Zoschke, C.; Wolff, C.; Darvin, M.E.; Sochorová, M.; Kováčik, A.; Wanjiku, B.; Schumacher, F.; Tigges, J.; Kleuser, B.; et al. Fibroblast origin shapes tissue homeostasis, epidermal differentiation, and drug uptake. *Sci. Rep.* **2019**, *9*, 1–10. [CrossRef] [PubMed]
3. Rogers, K.M.; Black, D.H.; Eberle, R. Primary mouse dermal fibroblast cell cultures as an in vitro model system for the differential pathogenicity of cross-species herpesvirus papio 2 infections. *Arch. Virol.* **2006**, *152*, 543–552. [CrossRef] [PubMed]
4. Pham, P.H.; Leacy, A.; Deng, L.; Nagy, É.; Susta, L. Isolation of Ontario aquatic bird bornavirus 1 and characterization of its replication in immortalized avian cell lines. *Virol. J.* **2020**, *17*, 1–12. [CrossRef] [PubMed]
5. Coelho, J.C.; Giugliani, R. Fibroblasts of skin fragments as a tool for the investigation of genetic diseases: Technical recommendations. *Genet. Mol. Biol.* **2000**, *23*, 269–271. [CrossRef]
6. Vangipuram, M.; Ting, D.; Kim, S.; Diaz, R.; Schuele, B. Skin Punch Biopsy Explant Culture for Derivation of Primary Human Fibroblasts. *J. Vis. Exp.* **2013**, e3779. [CrossRef] [PubMed]
7. Takashima, A. Establishment of Fibroblast Cultures. *Curr. Protoc. Cell Biol.* **1998**, 2.1.1–2.1.12. [CrossRef] [PubMed]
8. Khan, M.; Gasser, S. Generating Primary Fibroblast Cultures from Mouse Ear and Tail Tissues. *J. Vis. Exp.* **2016**, e53565. [CrossRef] [PubMed]
9. Ishak, M.F.; Manira, M.; Ng, M.H.; Khairul, B.; Gargy, L.; Aminuddin, B.S.; Ruszymah, B.H. Long Term Effect of Cryopreservation on Primary Human Skin Cells. *Sains Malays.* **2019**, *48*, 137–144. [CrossRef]
10. Siengdee, P.; Klinhom, S.; Thitaram, C.; Nganvongpanit, K. Isolation and culture of primary adult skin fibroblasts from the Asian elephant (*Elephas maximus*). *PeerJ* **2018**, *6*, e4302. [CrossRef] [PubMed]
11. Villegas, J.; McPhaul, M. Establishment and Culture of Human Skin Fibroblasts. *Curr. Protoc. Mol. Biol.* **2005**, *71*, 28.3.1–28.3.9. [CrossRef] [PubMed]
12. Moresco, K.A.; Stallknecht, D.E.; Swayne, D. Evaluation and Attempted Optimization of Avian Embryos and Cell Culture Methods for Efficient Isolation and Propagation of Low Pathogenicity Avian Influenza Viruses. *Avian Dis.* **2010**, *54*, 622–626. [CrossRef] [PubMed]
13. Kang, Y.; Feng, M.; Zhao, X.; Dai, X.; Xiang, B.; Gao, P.; Li, Y.; Li, Y.; Ren, T. Newcastle disease virus infection in chicken embryonic fibroblasts but not duck embryonic fibroblasts is associated with elevated host innate immune response. *Virol. J.* **2016**, *13*, 1–10. [CrossRef] [PubMed]
14. Kondo, A. Growth characteristics of rabies virus in primary chick embryo cells. *Virology* **1965**, *27*, 199–204. [CrossRef]
15. Ono, E.; Amagai, K.; Taharaguchi, S.; Tomioka, Y.; Yoshino, S.; Watanabe, Y.; Cherel, P.; Houdebine, L.-M.; Adam, M.; Eloit, M.; et al. Transgenic mice expressing a soluble form of porcine nectin-1/herpesvirus entry mediator C as a model for pseudorabies-resistant livestock. *Proc. Natl. Acad. Sci. USA* **2004**, *101*, 16150–16155. [CrossRef] [PubMed]
16. Gupta, S.; Gupta, N.; Ahlawat, S.; Kumar, A.; Taneja, R.; Sharma, R.; Sunder, S.; Tantia, M. In Vitro Culture of Skin Fibroblast Cells for Potential Cloning by Nuclear Transfer. In *Applications of Gene-Based Technologies for Improving Animal Production and Health in Developing Countries*; Springer: Dordrecht, The Netherlands, 2005; pp. 631–640. [CrossRef]

What is the meaning of viral nucleic acid detection in a host sample?

Scientific publication 3

Harmless or Threatening? Interpreting the Results of Molecular Diagnosis in the Context of Virus-Host Relationships

■ ■

Molecular methods are powerful tools to detect viruses' genomes and provide rapid diagnostic results. However, the meaning of these detections should be considered in all investigations

Harmless or Threatening? Interpreting the Results of Molecular Diagnosis in the Context of Virus-Host Relationships

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Molecular methods, established in the 1980s, expanded and delivered tools for the detection of vestigial quantities of nucleic acids in biological samples. Nucleotide sequencing of these molecules reveals the identity of the organism it belongs to. However, the implications of such detection are often misinterpreted as pathogenic, even in the absence of corroborating clinical evidence. This is particularly significant in the field of virology where the concepts of commensalism, and other benign or neutral relationships, are still very new. In this manuscript, we review some fundamental microbiological concepts including commensalism, mutualism, pathogenicity, and infection, giving special emphasis to their application in virology, in order to clarify the difference between detection and infection. We also propose a system for the correct attribution of terminology in this context.

Keywords: host-pathogen, detection, infection, commensalism, mutualism, molecular biology interpretation

INTRODUCTION

For centuries, the diagnosis of an infectious disease was solely based on clinical history and presentation. The first laboratory technique used to visualise microbes was microscopy (Gest et al., 2004). By the 19th century, the relationship between disease and pathogens was established, triggering a cascade of microbiological lines of research during multiple epidemics such as smallpox, diphtheria, tuberculosis, cholera, among others. This was pioneered by Robert Koch, who formulated strict criteria to determine the cause-effect relationship between a microbe and a disease: (i) The microorganism must be found in diseased but not healthy individuals, (ii) the microorganism must be cultured from the diseased individual, (iii) the inoculation of a healthy individual with cultured microorganism must induce disease, and (iv) the microorganism re-isolated from the inoculated individual may match with the original. This clear set of rules has since become blurred with increasing understanding of the spectrum of relationships between an organism and its host, ranging from mutualism to parasitism.

The ambiguity regarding the microbe-host relationship has been further amplified following the massive explosion of alternative molecular detection methods, such as the polymerase chain reaction (PCR) (Mullis et al., 1992), which provided the scientific community with new revolutionary powerful tools to rapidly identify new organisms and genetic diseases. More recently,

next generation sequencing (NGS) high throughput technology, which enables rapid sequencing of billions of DNA nucleotides, has enabled the study of microbiomes through the application of whole-genome sequencing (WGS) on microbial communities (metagenomics). The microbiome encompasses all the microorganisms living in or on any vertebrate animal, and can be sub-classified into the bacteriome, virome and mycobiome. The virome is composed of the collection of viruses that inhabit an organism (Lecuit and Eloit, 2013). In mammals, this includes viruses that infect the host, endogenous ancient virus-derived elements inserted in chromosomes, and viruses that infect members of the host's microbiome, like the phages that replicate in bacteria (Virgin, 2014; Cadwell, 2015). Compared to bacteriomes, human and animal viromes are less well known. The study of viromes is hindered by several technical limitations, namely the lack of common markers for viruses, the huge heterogeneity of the virome components, the difficulties of working with small samples, the contamination of the samples by host DNA, the lack of adequate bioinformatic tools for analysis and the absence of robust, refined and updated databases (Zou et al., 2016).

Since then, methods such as DNA microarray and genome sequencing led to the detection of microbes whose pathogenic potential was unapparent (Young et al., 2015). Some unexpected viruses have been found in samples from both healthy and immunosuppressed patients without signs of overt disease (Sauvage et al., 2011; Kapusinszky et al., 2012). Others have even been found to have beneficial effects on human and animal health due to their ability to influence the structure and function of bacterial communities through prokaryotic viruses (Sandaa et al., 2018). The ability to detect a tremendous variety of viruses with unclear pathogenic potential (**Table 1**) has re-emphasised the importance of an accurate description of the symbiosis.

Our perception of the role of viruses has shifted from solely sources of acute, persistent, or latent infections to commensal or even mutualistic organisms. The intricacy of virus-host relationships is reflected in the human genome composition, of which 5–8% is constituted by endogenous retroviruses (ERVs) (Nelson et al., 2004).

Today, molecular biology is an invaluable tool for diagnosis and research. However, the molecular detection of a potential pathogen in an animal or human can be easily misinterpreted. In the absence of corroborating clinical evidence, molecular detection often results in the assumption of an infection by default. This has become increasingly relevant in the field of virology, since the discovery of commensal and mutualistic viruses. The isolation and identification of a potential pathogen must be evaluated alongside the context of the microbial community to which it belongs to and the clinical evidence to suggest its interaction with the host.

In this manuscript, we review some fundamental microbiological concepts and explore how continuous discoveries in the field of microbiology demand some degree of re-framing of these concepts. We also discuss the strengths and weaknesses of several molecular diagnostic methods, exploring the differing implications of positive results. Finally, we propose a system for the correct attribution of terminology in this context.

TABLE 1 | Examples of commensal viruses.

Family	Evidence of colonisation
Anelloviridae	They are ubiquitous within the human species and have not yet been causally linked to any disease (Hino and Miyata, 2007; De Villiers and Zur Hausen, 2009). These viruses have been found in various organs, tissues and cell types (Tajiri et al., 2001; Thom et al., 2003), including plasma where they can cause persistent viraemia in 70% of worldwide population (Hino and Miyata, 2007)
Papillomaviridae *Polyomaviridae* *Circoviridae*	Three predominant families of viruses are found to colonise the human skin. Similarly to the skin microbiome, the skin human virome is composed of both resident and transient viruses (Lecuit and Eloit, 2013). High seropositivity (90%) to at least one human papilloma virus type has been reported in the human skin (Antonsson, 2012). Merkel cell polyomavirus, human polyomavirus 6, 7, and 9, are considered skin-tropic polyomaviruses existing chronically in healthy individuals (Feltkamp et al., 2013). Seropositivity of capsid protein VP1, a major structural component of the polyomavirus, can be detected in nearly 100% of the human population (Ott et al., 2000). Cyclovirus and other Circoviridae members are found in skin surface of both human and animals with cross-species transmission appearing possible (Li et al., 2011)
Picornaviridae	In the gastrointestinal tract, persistent or intermittent shedding of enteric viruses from healthy people is well established Human enterovirus Witsø et al., 2006 and parechovirus Olijve et al., 2018 are excreted by a large fraction of children under the age of five without any evidence of association with disease (Witsø et al., 2006; Olijve et al., 2018). Additionally, human enterovirus type 3 and 4 have the pig as reservoir Smith and Purdy, 2013
Anelloviridae	Kapusinszky et al. (2012) observed nearly constant shedding of anelloviruses including torque teno viruses Torque teno virus viraemia can be identified in nearly all individuals

THE SPECTRUM OF HOST-ORGANISM RELATIONSHIPS

The initial definitions underlying most of the concepts of microbiology were largely pathogen-centred. Later, the recognition that many microbial agents may interact with certain hosts without causing disease led to the establishment of new terminology to describe the distinct situations where this might happen. Social relationships between organisms can be very complex. The same organism can engage in different types of biological relationships with other organisms or hosts and follow co-evolutionary paths.

Below, we describe some of the most common symbiotic relationships between microorganisms and their hosts in which at least one of the partners involved in the interaction benefits from this close relationship.

Symbiotic relationships between animals and microorganisms are common and well-known, although terms like commensalism, mutualism and parasitism, referring to different types of symbiotic relationships are sometimes misunderstood. In virology, these terms are largely underused.

Commensalism

Commensalism describes the relationship between two organisms where one partner benefits whilst the other remains

unaffected. Mutualism describes a relationship in which both partners take advantage (win-win relationship) and the term "parasitism" refers to the case where one partner takes advantage over the other (win-lose relationship). This is often applied when the invading organisms produce harm to the host – infection (Fierer et al., 2017).

One could argue that viruses are by nature intracellular parasites, given that they rely on the high-jacking of cellular processes to replicate. In fact, it was believed that the normal cellular function would be disturbed in this process, leading inevitably to emergence of disease (Griffiths, 1999). As such it is unsurprising that the term "commensal" was never used in the same way in virology (Mims et al., 1998). However, Griffiths (1999) proposed the concept of "commensal viruses," suggesting they might remain within their host in a low replicative phase without therefore causing virus-induced cytolysis. For example, whilst an organism can be part of the natural and healthy microbiota without triggering any infectious disease, it can still pose the threat of pathogenicity. Threat as it can increase in number in the microbial community and lead to the onset of an infection. Because symbiotic relationships are dynamic and evolve over time, the imbalance of the bacterial diversity and load (dysbiosis) can become detrimental to the host (Zhang et al., 2015), and may promote opportunistic infections. Such is the case in the onset of pseudomembranous colitis following antibiotic therapeutic protocols, due to the overgrowth of the bacterial opportunistic pathogen *Clostridium difficile* in the human gut (David et al., 2019; Nogueira et al., 2019). Similarly, the overgrowth of the commensal yeast *Candida albicans* can result in oral thrush and oesophagitis (Deepa et al., 2014).

Mutualism

Microbiota that reside in the epithelial tissue that is exposed to the external environment in the respiratory, gastrointestinal and vaginal tracts as well as in the skin since birth are often called commensal (Tlaskalová-hogenová et al., 2004), suggesting that neither it, nor its host, benefit or suffer from its presence. However, extensive research on the effect of microbiota on human and animal health has highlighted the presence of many symbiotic relationships between microorganisms and the host, generally beneficial to the host, and therefore better described as a type of mutualistic relationship (Macpherson and Mccoy, 2014).

The bacteria composing the human gut microbiota supply vitamins, aid in digestion of carbohydrates, maintain the integrity of mucosal barrier, and prevent overgrowth and invasion of pathogenic bacteria (Zhang et al., 2015).

Microbiota imbalances have therefore been linked to many human diseases including inflammatory bowel diseases, cardiovascular disease, obesity, and type 2 diabetes (Malinen et al., 2005; Frank et al., 2007; Turnbaugh et al., 2009; Larsen et al., 2010; Gerritsen et al., 2011; Kerckhoffs et al., 2011).

Mutualistic relationships between viruses and their hosts have also been revealed. Some authors have suggested that highly prevalent viruses, such as herpesviruses, may actually play a protective role against bacterial infection by boosting innate immunity (Dickinson, 2018). Some mouse herpesviruses, highly similar to the human Epstein Barr virus (EBV) and

cytomegalovirus (CMV), activate the innate immune response and protect mice against bacteria (Barton et al., 2007). The γ-herpesvirus 68 (γHV68), for example, was found to protect against infection by *Listeria monocytogenes* and *Yersinia pestis* by sustaining IFN-γ production and macrophage activation (Barton et al., 2007). The murine norovirus can replace many of the benefits provided by commensal bacteria in the intestine (Kernbauer et al., 2014), and chronically it can lead to low expression of *Atg16L1*, an autophagy gene with allelic variants that predisposes to Crohn's disease. The surprising finding that gyroviruses encode a protein that is specifically cytotoxic to cancer cells, raises the possibility that some viral infections can be beneficial in controlling the development of tumour cells (Los et al., 2009), whilst others are directly causative of certain kinds of cancer, such as Burkitt's lymphoma and cervical cancer.

PATHOGENICITY, VIRULENCE, AND INFECTION

A pathogen is a microorganism that can cause damage to its host. Pathogenicity results from the expression of virulent factors, proteins which are essential for the invasion and colonisation of the host, evasion of its immune system and nutrient uptake at its expense. Infection is the damage inflicted on the host during this process.

Casadevall and Pirofski (2000) highlighted that a coloniser organism can cause varying degrees of damage to its host, from none to substantial. The latter effect induces host responses that might be successful in eliminating the microbe or might be unsuccessful, consequently progressing to chronic infection. For those organisms that, once having colonised the host, induce no damage, its state is indistinguishable from "commensalism" (Casadevall and Pirofski, 1999).

SARS-CoV-2, is an example of a pathogen that induces a range of symptoms. Although a significant proportion of people infected with this virus do not display any symptoms (Yanes-Lane et al., 2020), detection of the virus in these individuals is always referred to as an infection, albeit asymptomatic, given the clear association between the pathogen and respiratory disease it is capable of causing. These asymptomatic infections differ from commensal colonisation due to the complete elimination of the offending organism by the immune system.

In 1999, Casadevall and Pirofski revised the term "pathogen" to mean a microbe capable of causing damage to its host, to highlight what they believed was the most relevant outcome of the host-pathogen interaction (Casadevall and Pirofski, 2000). Injury can result from either direct microbial action or the host immune response, or often both, can usually be identified through a combination of symptomatology, clinical examination and histology. However, damage being inflicted at a cellular level may escape detection by these methods. For example, high-risk human papillomavirus (HPV) types, responsible for the vast majority of cervical cancers, inactivate the essential tumour suppressor genes *pRb* and *p53* in host cells in order to induce in them a perpetual replicative state, necessary for optimal viral replication (Buitrago-pérez et al., 2009). Silencing of tumour

suppressor genes in cervical cells does not immediate cause overt injury that is clinically or histologically identifiable. In fact, pre-cancerous dysplastic changes, can take a number of years to develop (Burd and Burd, 2003; Castle and Fetterman, 2009). Nonetheless, lack of evidence of damage does not indicate the offending organism is not a pathogen, when there is substantial reliable historical scientific evidence to the contrary.

Organisms can be described in terms of virulence and pathogenicity, which have been defined in various ways throughout the years, but overall describe the features or characteristics that enable an organism to cause disease and the degree or speed at which a pathogen can cause disease, respectively. Some concepts were recently revised by Casadevall and Pirofski (1999). These authors critically reviewed the origin and historical definitions of terms namely infection, commensalism, colonisation, persistence, infection, and disease and updated them in order to recognise current knowledge.

According to Lwoff (1957), "infection" is the introduction of a foreign entity that is capable of multiplying to produce additional infectious entities into an organism, regardless of whether this results in a disease. This definition encompasses the concept of subclinical or unapparent infections, which cause no signs or symptoms (Mahy, 2009).

Arguably, there are pitfalls to all definitions. Prions, infective proteins that can cause often devastating disease, might not technically be classified as microorganisms, and yet their pathogenicity is undeniable. This is also the case with infectious nucleic acid and infectious viral particles, which contain partial or complete viral genome. Some microorganisms pertaining to the healthy microbiota, although beneficial to their hosts much of the time, have the potential to cause disease though opportunistic infection. We rely on our microbiome to perform many human physiological functions, such as vitamin synthesis. Should these organisms be thought of as pathogens, as per Casadevall and Pirofski (1999), because they have the potential to cause damage? This seems to overlook their potential for beneficial effects. We do not agree with their revised definition of "infection," we would argue that "acquisition of a microbe by host" should preferably be defined instead as "colonisation" when the impact on the host is unknown. After this process of "acquisition," the pathophysiological sequence, it is often possible to predict and dependent on the pathotype – a group of organisms with same pathogenicity on a host. Only with evidence of pathogenesis caused by the colonisation of this organism, in an acute, chronic or intermittent manner, can the process be labelled an "infection." This distinction better reflects the implications of this microbiological process in clinical practice.

INEQUALITY OF DIAGNOSTIC TESTS: DETECTION VERSUS INFECTION

There is a growing range of widely available diagnostic methods capable of detecting an organism or potential pathogen.

Culture-based methods provide evidence of viable infectious pathogens in the sample by demonstrating the growth of organisms *in vitro*. This is not only applicable to the growth of bacteria in culture media, but also to the growth of viruses and intracellular bacteria in susceptible eukaryotic cell lines. Although the growth of bacteria can be observed with the naked eye or simple light microscopy, growth of intracellular bacteria and viruses can be confirmed through staining techniques or identification of specific virus-induced cytopathic effects. Electron microscopy (EM) can be used to identify both mature and immature forms of viral particles within a cell. The simultaneous presence of both forms indicates active viral replication. However, it has a much lower sensitivity than molecular methods and requires specialised technicians and equipment.

Antigen tests detect certain proteins of a specific organism through immunoassays. They are quick and achieve a high specificity by targeting proteins or specific epitopes that are singular to the pathogen being detected. However, antigen tests can have low sensitivities, and therefore a higher rate of false negative results, when compared to PCR. Rapid influenza diagnostic tests (RIDTs), for example, which detect influenza virus nucleoproteins, only achieve a sensitivity of around 50–80% (CDC, 2020b). COVID-19 lateral flow rapid antigen tests also vary in sensitivity from 79%, when performed by laboratory scientists, to 58%, when performed by self-trained members of the public (Mahase, 2020). Rapid antigen tests for pathogens like group A beta-haemolytic streptococci and Hepatitis B virus, on the other hand, have substantially higher sensitivities of 90% and above (Joslyn et al., 1995). The sensitivity of these tests is dependent on factors such as timing of sample collection, collection technique and viral load (Tanei et al., 2014).

Importantly, a viable virus is not required for detection of an antigen. In infections like COVID-19 where viral shedding continues beyond the resolution of infection, meaning there is the presence of virus particles but no actual viable virus, a positive antigen test cannot distinguish between active infection with transmission potential and resolved infection without transmission potential (Cevik et al., 2021). Antigen detection should, therefore, be interpreted as current *or* recent infection and should not infer infectiousness.

Antibodies are produced by the humoural immune system in response to the detection of an antigen and can arise from either natural infection or vaccination. A positive antibody test thus indicates that there has been, at some point, an exposure to the organism.

There are several ways to distinguish between active and past infection. Detection of IgM antibodies, which are produced as the first response to a new infection but are only short-term and start dropping a few weeks after infection, is likely to indicate a current or recent infection.

IgG antibodies, on the other hand, are produced later, in the course of the infection, and can remain in the bloodstream for months to years. Because the lag time between initial infection and antibody production, timing of this diagnostic test is crucial to avoid false negative results.

The detection of specific class of antibodies against non-structural viral proteins is indicative of active viral replication and is therefore also a useful tool to detect ongoing infection. Unlike non-structural protein, which are present in much larger

FIGURE 1 | Schematic representation of scientific process to classification of virus-host interaction.
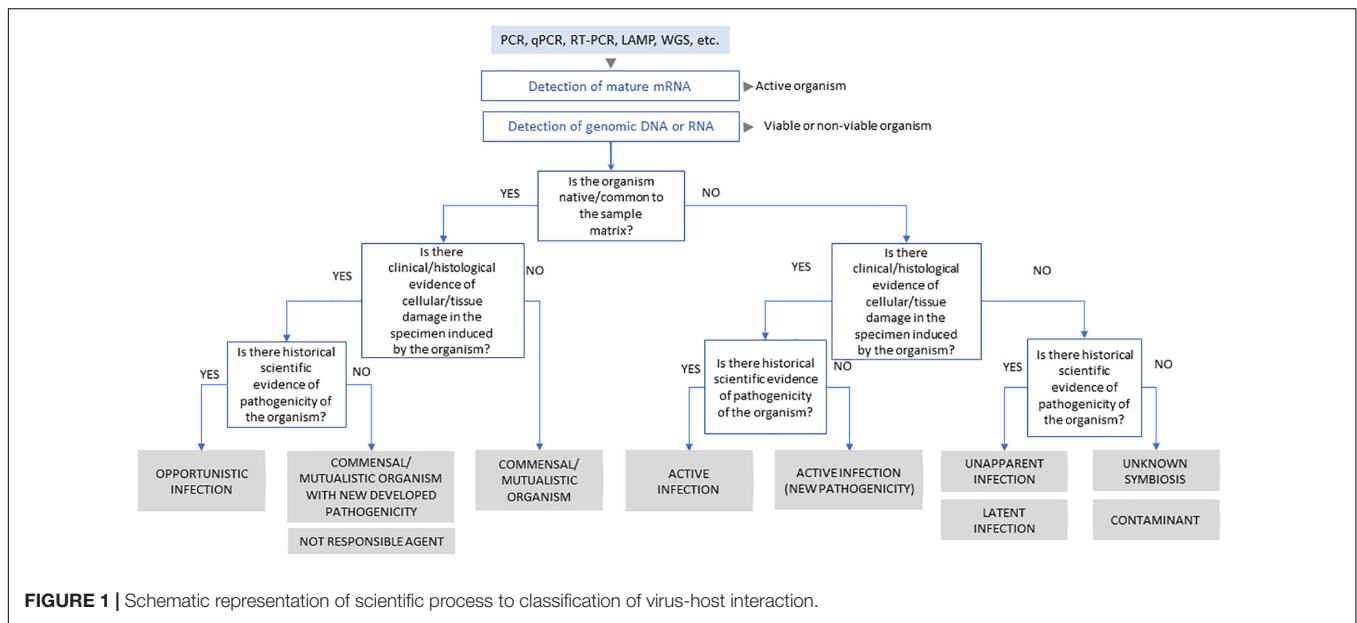
amounts, the structural proteins are fewer and less immunogenic. Their antibodies are thus short-lived and consequently un-detectable soon after the resolution of infection.

They have also been used in Differentiate Infected from Vaccinated Animals (DIVA) tests, given vaccines (with the exception of live and attenuated vaccines) do not result in the production of antibodies against non-structural proteins. This is the case with Hepatitis B. Surface antibodies can be produced in response to both active infection and as a result of vaccination, whereas core (non-structural) antibodies are only produced following natural infection.

Molecular based detection methods have some unquestionable advantages compared to the methods mentioned above, including their greater sensitivity, specificity and ability to be automated. However, the component that is being detected, whether that be genomic components or messenger RNA is paramount to the interpretation of a positive result.

Other group of molecular diagnostic tests detect genetic material that is specific an organism. The detection of mature messenger RNA provides evidence of active infection, as it implies gene expression, contrary to positivity by standard PCR, where detection may also represent the presence of nucleic acids from non-viable and therefore non-infectious organisms. The detection of mRNA can, therefore, help distinguish between viral latency and active replication (Lecuit and Eloit, 2013).

Although highly sensitive and specific, PCR test results are not always clear-cut with other interpretation issues such as the clinical significance of weaker signals, which represent a low copy number of a particular pathogen (Louie et al., 2000).

In situ hybridisation (ISH) detects viral genome in tissues or cells, by localisation of specific unique or repeated DNA and RNA sequences using complementary labelled probes. ISH demonstrates specific nucleic acid sequences in their cellular environment. As such, it can provide information regarding the level and place of mRNA expression, demonstrating the presence

of newly synthesised viral DNA or RNA within cells, hence confirming the pathogens viability.

Some examples of diverse interpretations of molecular results have been provided over the years in both the bacteriology and virology fields. In 2019 the Centre for Disease Control and Prevention (CDC) alerted to the fact that although rapid molecular assays including PCR and other alternative nucleic acid amplification methods can detect viral RNA in respiratory specimens with high sensitivity and specificity, this result does not necessarily indicate detection of a viable virus or on-going influenza viral replication (CDC, 2019). Similarly, the detection of cytomegalovirus DNA from a patient's serum cannot distinguish between active disease or latent infection (Ljungman et al., 2017), which are distinct situations from a clinical standpoint. Therefore, the use of sensitive laboratory techniques to test for the presence of novel viruses must be supported by additional clinical evidence to convincingly indicate that the detected virus was the cause of the observed disease (Griffiths, 1999).

The nuances of molecular biology result interpretation is a significant topic of discussion in the context of the recent global outbreak of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) (Suri et al., 2020). Multiple studies have described a positive RT-PCR result several days after recovery (Lan et al., 2020; Young et al., 2020). Other cases have reported positive RT-PCR results following several consecutive negative results. The clinical and infection-control implications of this are unclear. However, the general consensus is that viral RNA detection does not necessarily indicate the presence of active infection or transmissible viable viral particles (CDC, 2020a). In fact, RT-PCR detects RNA, not an infectious agent, limiting the ability to determine the infectiousness of patients or animals with a positive PCR (Bullard et al., 2020). It has been suggested that quantifying viral loads may help to clarify the likely clinical picture. One study correlated the success of viral isolates with viral loads,

and found that samples containing $<10^6$ copies per ml never yielded an isolate. Thus it concluded that despite the detection of viral loads long after symptom resolution there would be little residual risk of infection (Wölfel et al., 2020). Given the general ambiguity around infective potential of recovered patients, future focus on detection of mRNA throughout the course of the disease, rather than RNA, might produce more elucidating results. This COVID-19 pandemic has highlighted the importance of correct interpretation of molecular biology test results given its potential to influence global guidance on appropriate time of patient discharge and isolation length.

CONCLUSION

Molecular biology, biotechnology, genomics, and bioinformatics were the basis for one of the most important revolutions in recent microbiology, providing a boom of different conceptual methods that quickly replaced most of the classic, time-consuming, and laborious laboratory techniques used for the diagnosis of microbiological diseases. This revolution represented a change of focus from the agent itself to the simple identification of nucleic acids. However, contrary to the method of culture and isolation in cell lines, the detection of nucleic acid does not necessarily indicate the presence of viable organisms capable of replication and infection, given viral particles can persist even after resolution of infection. Other methods aimed at detecting messenger RNA or non-structural proteins on the other hand can reliably indicate an active infection.

Molecular biology is not only enabling the identification of new viruses but also the genotyping and viral load quantification of these organisms. However, virology has lagged in the exploration of the different types of virus-host relationships. For viruses, whose pathogenic virulence is recognised, the characterisation of virus-host relationships is simple. Nonetheless, a growing number of non-pathogenic

viruses establish states of commensalism or mutualism with their hosts. At times, key features of these complex microbiological states and processes overlap, impairing recognition and classification. This issue is further exacerbated by the nature of most investigations, which are not longitudinal and therefore cannot capture such dynamics.

Thus, we emphasise the importance of combining physiopathological evidence to the molecular data when describing the novel presence of a microorganism in a host. Where this is not possible, we suggest the use of more conservative language which avoids charged terms such as "infection" and "pathogen." To aid the appropriate use of what should be standardised terminology, we propose a rationale to characterise microbe-host relationships (**Figure 1**).

AUTHOR CONTRIBUTIONS

FAAS, SP, and TN: conceptualisation and writing – original draft preparation. CC: writing – original draft preparation. RS: writing – review and editing. MD: conceptualisation and writing – review and editing. All authors contributed to the article and approved the submitted version.

REFERENCES

Antonsson, A. (2012). Review: antibodies to cutaneous human papillomaviruses. *J. Med. Virol.* 84, 814–822. doi: 10.1002/jmv

Barton, E. S., White, D. W., Cathelyn, J. S., Brett-mcclellan, K. A., Engle, M., Diamond, M. S., et al. (2007). Herpesvirus latency confers symbiotic protection from bacterial infection. *Nature* 447, 326–329. doi: 10.1038/nature05762

Buitrago-pérez, Á, Garaulet, G., Vázquez-carballo, A., and Paramio, J. M. (2009). Molecular signature of hpV-Induced carcinogenesis: pRb, p53 and gene expression profiling. *Curr. Genomics* 10, 26–34. doi: 10.2174/138920209787581235

Bullard, J., Dust, K., Funk, D., Strong, J. E., Alexander, D., Garnett, L., et al. (2020). Predicting infectious SARS-CoV-2 from diagnostic samples. *Clin. Infect. Dis. Off. Publ. Infect. Dis. Soc. Am.* 71, 2663–2666. doi: 10.1093/cid/ciaa638

Burd, E. M., and Burd, E. M. (2003). Human papillomavirus and cervical cancer. *Clin. Microbiol. Rev.* 16, 1–17. doi: 10.1128/CMR.16.1.1

Cadwell, K. (2015). The virome in host health and disease. *Immunity* 42, 805–813. doi: 10.1016/j.immuni.2015.05.003

Casadevall, A., and Pirofski, L. (2000). MINIREVIEW host-pathogen interactions: basic concepts of microbial commensalism, colonization, infection, and disease. *Infect. Immun.* 68, 6511–6518. doi: 10.1128/IAI.68.12.6511-6518.2000.Updated

Casadevall, A., and Pirofski, L. A. (1999). Host-pathogen interactions: redefining the basic concepts of virulence and pathogenicity. *Infect. Immun.* 67, 3703–3713. doi: 10.1128/iai.67.8.3703-3713.1999

Castle, P. E., and Fetterman, B. (2009). Five-Year experience of human papillomavirus DNA and papanicolaou test cotesting. *Obstet. Gynecol.* 113, 595–600. doi: 10.1097/aog.0b013e3181996ffa

CDC (2019). *Information on Rapid Molecular Assays, RT-PCR, and other Molecular Assays for Diagnosis of Influenza Virus Infection.* Available online at: https://www.cdc.gov/flu/professionals/diagnosis/molecular-assays.htm (accessed April 1, 2020).

CDC (2020a). *Clinical Questions about COVID-19: Questions and Answers.* Available online at: https://www.cdc.gov/coronavirus/2019-ncov/hcp/faq.html (accessed August 30, 2020).

CDC (2020b). *Overview of Influenza Testing Methods. Centers Dis. Control Prev. Natl. Cent. Immun. Respir. Dis.* Available online at: https://www.cdc.gov/flu/professionals/diagnosis/overview-testing-methods.htm (accessed November 13, 2020).

Cevik, M., Tate, M., Lloyd, O., Maraolo, A. E., Schafers, J., and Ho, A. (2021). SARS-CoV-2, SARS-CoV, and MERS-CoV viral load dynamics, duration of viral shedding, and infectiousness: a systematic review and meta-analysis. *Lancet Microbe* 2, e13–e22. doi: 10.1016/s2666-5247(20)30172-5

David, P., Sá-pinto, X., and Nogueira, T. (2019). Microbiomes are important key players in human health-mutualistic interactions with and between our microbes. *JOJ Int. Med.* 1:555556.

De Villiers, E.-M., and Zur Hausen, H. eds (2009). *TT viruses: The Still Elusive Human Pathogens.* Berlin: Springer.

Deepa, A., Nair, B. J., Sivakumar, T., and Joseph, A. P. (2014). Uncommon opportunistic fungal infections of oral cavity: a review. *J. Oral Maxillofac. Pathol.* 18, 235–243. doi: 10.4103/0973-029X.140765

Dickinson, A. M. (2018). Potential beneficial effects of cytomegalovirus infection after transplantation. *Front. Immunol.* 9:389. doi: 10.3389/fimmu.2018.00389

Feltkamp, M. C. W., Kazem, S., Van Der Meijden, E., Lauber, C., and Gorbalenya, A. E. (2013). From Stockholm to Malawi: recent developments in studying human polyomaviruses. *J. Gen. Virol.* 94, 482–496. doi: 10.1099/vir.0.048462-0

Fierer, J., Looney, D., and Pechère, J.-C. (2017). Nature and Pathogenicity of Micro-organisms. *Infect. Dis. (Auckl).* 1, 4–25.e1. doi: 10.1016/b978-0-7020-6285-8.00002-2

Frank, D. N., St. Amand, A. L., Feldman, R. A., Boedeker, E. C., Harpaz, N., and Pace, N. R. (2007). Molecular-phylogenetic characterization of microbial community imbalances in human inflammatory bowel diseases. *Proc. Natl. Acad. Sci. U.S.A.* 104, 13780–13785. doi: 10.1073/pnas.0706625104

Gerritsen, J., Smidt, H., Rijkers, G. T., and De Vos, W. M. (2011). Intestinal microbiota in human health and disease: the impact of probiotics. *Genes Nutr.* 6, 209–240. doi: 10.1007/s12263-011-0229-7

Gest, H., Leeuwenhoek, A. V. A. N., Of, F., and Royal, T. H. E. (2004). The discovery of microorganisms by Robert Hooke and Antoni van Leeuwenhoek, Fellows of The Royal Society. *Notes Rec. R. Soc. Lond.* 58, 187–201. doi: 10.1098/rsnr.2004.0055

Griffiths, P. (1999). Time to consider the concept of a commensal virus? *Rev. Med. Virol.* 9, 73–74. doi: 10.1002/(sici)1099-1654(199904/06)9:2<73::aid-rmv254>3.0.co;2-5

Hino, S., and Miyata, H. (2007). Torque teno virus (TTV): current status. *Rev. Med. Virol.* 17, 45–57. doi: 10.1002/rmv.524

Joslyn, S. A., Hoekstra, G. L., and Sutherland, J. E. (1995). Rapid antigen detection testing in diagnosing group A beta-hemolytic streptococcal pharyngitis. *J. Am. Board Fam. Pract.* 8, 177–182.

Kapusinszky, B., Minor, P., and Delwart, E. (2012). Nearly constant shedding of diverse enteric viruses by two healthy infants. *J. Clin. Microbiol.* 50, 3427–3434. doi: 10.1128/JCM.01589-12

Kerckhoffs, A. P. M., Ben-Amor, K., Samsom, M., Van Der Rest, M. E., De Vogel, J., Knol, J., et al. (2011). Molecular analysis of faecal and duodenal samples reveals significantly higher prevalence and numbers of *Pseudomonas aeruginosa* in irritable bowel syndrome. *J. Med. Microbiol.* 60, 236–245. doi: 10.1099/jmm.0.022848-0

Kernbauer, E., Ding, Y., and Cadwell, K. (2014). An enteric virus can replace the beneficial function of commensal bacteria. *Nature* 516, 94–98. doi: 10.1038/nature13960

Lan, L., Xu, D., Ye, G., Xia, C., Wang, S., Li, Y., et al. (2020). Positive RT-PCR test results in patients recovered from COVID-19. *JAMA* 323, 1502–1503. doi: 10.1001/jama.2020.2783 .

Larsen, N., Vogensen, F. K., Van Den Berg, F. W. J., Nielsen, D. S., Andreasen, A. S., Pedersen, B. K., et al. (2010). Gut microbiota in human adults with type 2 diabetes differs from non-diabetic adults. *PLoS One* 5:9085. doi: 10.1371/journal.pone.0009085

Lecuit, M., and Eloit, M. (2013). The human virome: new tools and concepts. *Trends Microbiol.* 21, 510–515. doi: 10.1016/j.tim.2013.07.001

Li, L., Shan, T., Soji, O. B., Alam, M., Kunz, T. H., Zaidi, S. Z., et al. (2011). Communication Possible cross-species transmission of circoviruses and cycloviruses among farm animals. *J. Gen. Virol.* 92, 768–772. doi: 10.1099/vir.0.028704-0

Ljungman, P., Boeckh, M., Hirsch, H. H., Josephson, F., Lundgren, J., Nichols, G., et al. (2017). Definitions of cytomegalovirus infection and disease in transplant patients for use in clinical trials. *Clin. Infect. Dis. Off. Publ. Infect. Dis. Soc. Am.* 64, 87–91. doi: 10.1093/cid/ciw668

Los, M., Panigrahi, S., Mandal, S., Stetefeld, J., Essmann, F., and Schulze-osthoff, K. (2009). Biochimica et Biophysica Acta Apoptin, a tumor-selective killer. *BBA Mol. Cell Res.* 1793, 1335–1342. doi: 10.1016/j.bbamcr.2009.04.002

Louie, M., Louie, L., and Simor, A. E. (2000). The role of DNA amplification technology in the diagnosis of infectious diseases. *CMAJ* 163, 301–309. doi: 10.1016/s1381-1169(00)00220-x

Lwoff, A. (1957). The concept of virus. *J. Gen. Microbiol.* 17, 239–253. doi: 10.1099/00221287-17-2-239

Macpherson, A. J., and Mccoy, K. D. (2014). Standardised animal models of host microbial mutualism. *Mucosal Immunol.* 8, 476–486. doi: 10.1038/mi.2014.113

Mahase, E. (2020). Covid-19: innova lateral flow test is not fit for "test and release" strategy, say experts. *BMJ* 371:m4469. doi: 10.1136/bmj.m4469

Mahy, B. W. J. (2009). *Dictionary of Virology*. London: Elsevier.

Malinen, E., Rinttilä, T., Kajander, K., Mättö, J., Kassinen, A., Krogius, L., et al. (2005). Analysis of the fecal microbiota of irritable bowel syndrome patients and healthy controls with real-time PCR. *Am. J. Gastroenterol.* 100, 373–382. doi: 10.1111/j.1572-0241.2005.40312.x

Mims, C., Playfair, J., and Roitt, I. (1998). "The host–parasite response," in *Medical Microbiology*, eds I. R. C. Mims and J. Playfair (London: Mosby), 9–20.

Mullis, K., Faloona, F., Scharf, S., Saiki, R., Horn, G., and Erlich, H. (1992). Specific enzymatic amplification of DNA in vitro: the polymerase chain reaction. 1986. *Biotechnology* 24, 17–27.

Nelson, P. N., Hooley, P., Roden, D., Ejtehadi, H. D., Rylance, P., Warren, P., et al. (2004). Human endogenous retroviruses: transposable elements with potential? *Clin. Exp. Immunol.* 138, 1–9. doi: 10.1111/j.1365-2249.2004.02592.x

Nogueira, T., David, P. H. C., and Pothier, J. (2019). Antibiotics as both friends and foes of the human gut microbiome: the microbial community approach. *Drug Dev. Res.* 80, 86–97. doi: 10.1002/ddr.21466

Olijve, L., Jennings, L., and Walls, T. (2018). crossm human parechovirus: an increasingly recognized cause of sepsis-like illness in young infants. *Clin. Microbiol. Rev.* 31, 1-17. doi: 10.1128/CMR.00047-17

Ott, C., Duret, L., Chemin, I., Tre, C., Mandrand, B., Komurian-pradel, F., et al. (2000). Use of a TT virus ORF1 recombinant protein to detect anti-TT virus antibodies in human sera. *J. Gen. Virol.* 81, 2949–2958. doi: 10.1099/0022-1317-81-12-2949

Sandaa, R. A., Storesund, J. E., Olesin, E., Paulsen, M. L., Larsen, A., Bratbak, G., et al. (2018). Seasonality drives microbial community structure, shaping both eukaryotic and prokaryotic host–viral relationships in an arctic marine ecosystem. *Viruses* 10:715. doi: 10.3390/v10120715

Sauvage, V., Cheval, J., Foulongne, V., Gouilh, M. A., Pariente, K., Manuguerra, J. C., et al. (2011). Identification of the first human gyrovirus, a virus related to chicken anemia virus. *J. Virol.* 85, 7948–7950. doi: 10.1128/jvi.00639-11

Smith, D. B., and Purdy, M. A. (2013). Genetic variability and the classification of hepatitis E virus. *J. Virol.* 87, 4161–4169. doi: 10.1128/JVI.02762-12

Suri, T., Mittal, S., Tiwari, P., Mohan, A., Hadda, V., Madan, K., et al. (2020). COVID-19 real-time RT-PCR: does positivity on follow-up RT-PCR always imply infectivity? *Am. J. Respir. Crit. Care Med.* 202:147. doi: 10.1164/rccm.202004-1287LE

Tajiri, H., Tanaka, T., Sawada, A., Etani, Y., Kozaiwa, K., Mushiake, S., et al. (2001). Three cases with TT virus infection and idiopathic neonatal hepatitis. *Intervirology* 44, 364–369. doi: 10.1159/000050073

Tanei, M., Yokokawa, H., Murai, K., Sakamoto, R., Amari, Y., Boku, S., et al. (2014). Factors influencing the diagnostic accuracy of the rapid influenza antigen detection test (RIADT): a cross-sectional study. *BMJ Open* 4, 6–11. doi: 10.1136/bmjopen-2013-003885

Thom, K., Morrison, C., Lewis, J. C. M., and Simmonds, P. (2003). Distribution of TT virus (TTV), TTV-like minivirus, and related viruses in humans and nonhuman primates. *Virology* 306, 324–333. doi: 10.1016/S0042-6822(02)00049-1

Tlaskalová-hogenová, H., Štˇ, R., Cukrowska, B., Lodinová-žádn, R., Kozáková, H., Rossmann, P., et al. (2004). Commensal bacteria (normal microflora), mucosal immunity and chronic inflammatory and autoimmune diseases. *Immunol Lett.* 93, 97–108. doi: 10.1016/j.imlet.2004.02.005

Turnbaugh, P. J., Hamady, M., Yatsunenko, T., Cantarel, B. L., Ley, R. E., Sogin, M. L., et al. (2009). A core gut microbiome between lean and obesity twins. *Nature* 457, 480–484. doi: 10.1038/nature07540.A

Virgin, H. W. (2014). The virome in mammalian physiology and disease. *Cell* 157, 142–150. doi: 10.1016/j.cell.2014.02.032

Witsø, E., Palacios, G., Cinek, O., Stene, L. C., Grinde, B., Janowitz, D., et al. (2006). High prevalence of human enterovirus a infections in natural circulation of human enteroviruses. *J. Clin. Microbiol.* 44, 4095–4100. doi: 10.1128/JCM.00653-06

Wölfel, R., Corman, V. M., Guggemos, W., Seilmaier, M., Zange, S., Müller, M. A., et al. (2020). Virological assessment of hospitalized patients with COVID-2019. *Nature* 581, 465–469. doi: 10.1038/s41586-020-2196-x

Yanes-Lane, M., Winters, N., Fregonese, F., Bastos, M., Perlman-Arrow, S., Campbell, J. R., et al. (2020). Proportion of asymptomatic infection among COVID-19 positive persons and their transmission potential: a systematic review and meta-analysis. *PLoS One* 15:e0241536. doi: 10.1371/journal.pone.0241536

Young, B. E., Ong, S. W. X., Kalimuddin, S., Low, J. G., Tan, S. Y., Loh, J., et al. (2020). Epidemiologic features and clinical course of patients infected with SARS-CoV-2 in Singapore. *JAMA* 323, 1488–1494.

Young, J. C., Chehoud, C., Bittinger, K., Bailey, A., Diamond, J. M., Cantu, E., et al. (2015). Viral metagenomics reveal blooms of anelloviruses in the respiratory tract of lung transplant recipients. *Am. J. Transplant.* 15, 200–209. doi: 10.1111/ajt.13031

Zhang, Y., Li, S., Gan, R., Zhou, T., Xu, D., and Li, H. (2015). Impacts of gut bacteria on human health and diseases. *Int. J. Mol. Sci.* 16, 7493–7519. doi: 10.3390/ijms16047493

Zou, S., Caler, L., Colombini-Hatch, S., Glynn, S., and Srinivas, P. (2016). Research on the human virome: where are we and what is next. *Microbiome* 4:32. doi: 10.1186/s40168-016-0177-y

How to differentiate the new recombinant strain (ha-MYXV) from all classic strains?

Scientific publication 4

A Quadruplex qPCR for Detection and Differentiation of Classic and Natural Recombinant Myxoma Virus Strains of Leporids

■■■

In autumn 2018, a natural recombinant myxoma virus emerged in Portugal in Iberian hares and a year later in domestic and wild rabbits. The rapid diagnosis and differentiation of these strains allows epidemiological monitoring and the establishment of surveillance and control measures, with Quadruplex qPCR being the only method currently available that can be used in this context.

Article

A Quadruplex qPCR for Detection and Differentiation of Classic and Natural Recombinant Myxoma Virus Strains of Leporids

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Abstract: A natural recombinant myxoma virus (referred to as ha-MYXV or MYXV-Tol08/18) emerged in the Iberian hare (*Lepus granatensis*) and the European rabbit (*Oryctolagus cuniculus*) in late 2018 and mid-2020, respectively. This new virus is genetically distinct from classic myxoma virus (MYXV) strains that caused myxomatosis in rabbits until then, by acquiring an additional 2.8 Kbp insert within the *m009L* gene that disrupted it into ORFs *m009L-a* and *m009L-b*. To distinguish ha-MYXV from classic MYXV strains, we developed a robust qPCR multiplex technique that combines the amplification of the m000.5L/R duplicated gene, conserved in all myxoma virus strains including ha-MYXV, with the amplification of two other genes targeted by the real-time PCR systems designed during this study, specific either for classic MYXV or ha-MYXV strains. The first system targets the boundaries between ORFs *m009L-a* and *m009L-b*, only contiguous in classic strains, while the second amplifies a fragment within gene *m060L*, only present in recombinant MYXV strains. All amplification reactions were validated and normalized by a fourth PCR system directed to a housekeeping gene (*18S rRNA*) conserved in eukaryotic organisms, including hares and rabbits. The multiplex PCR (mPCR) technique described here was optimized for *Taqman*® and *Evagreen*® systems allowing the detection of as few as nine copies of viral DNA in the sample with an efficiency > 93%. This real-time multiplex is the first fast method available for the differential diagnosis between classic and recombinant MYXV strains, also allowing the detection of co-infections. The system proves to be an essential and effective tool for monitoring the geographical spread of ha-MYXV in the hare and wild rabbit populations, supporting the management of both species in the field.

Keywords: molecular diagnosis; multiplex; quadruplex qPCR; real-time PCR; *myxoma virus*; MYXV; natural recombinant MYXV; ha-MYXV; Iberian hare; European rabbit

1. Introduction

Planet Earth has around 34 global diversity hotspots, featuring exceptional concentrations of endemic species, of uncountable and indisputable value [1,2]. For the past several decades, particularly after 1970, these areas have been experiencing an exceptional loss of habitats [3]. The decline or even disappearance of key species from these hotspots has been identified as the main threat, leading to catastrophic cascading effects on the affected ecosystems [4]. The western Mediterranean Basin hotspot holds a particularly high taxon diversity, at a global level, only overpassed in importance by the tropics [5]. In fact, in this area one can find the same plant richness (30,000 taxa) of all tropical Africa,

which presents an area four times larger. In the western Mediterranean Basin hotspot 10.8 species/1000 km^2 can be found, a much higher value than the homologous number in countries such as China, Zaire, India or Brazil [6]. The relevance of the wild rabbit (*Oryctolagus cuniculus*) for the maintenance of the Mediterranean scrubland of south-western Europe is such, that some ecologists named this ecosystem "the rabbit's ecosystem" [7]. The wild rabbit and the Iberian hare (*Lepus granatensis*) are two of the most iconic and important species of the Iberian Peninsula, where they play a unique ecologic, cultural, and economic role.

Myxoma virus is a *Leporipoxvirus* from *Chordopoxvirinae* subfamily and *Poxviridae* family [8]. The myxoma virus (MYXV) possesses a large, linear double-stranded DNA genome with terminal inverted repeats (TIRs) and covalently closed hairpin loops at each end [9]. The Lausanne strain, the reference strain of classic Myxoma virus, encodes 158 unique open reading frames (ORFs), 12 being duplicated in the 11,577 bp-long TIRs [10,11].

In late 2018, a natural recombinant *myxoma virus* (hereinafter referred to as ha-MYXV, but also known as MYXV-Tol08/18) emerged in the Iberian hare (*Lepus granatensis*), affecting several populations in Spain [12–14] and Portugal [15] with an apparent mean mortality rate of 55.4% [14], and remarkable geographic dissemination, extending to all the Iberian peninsula in the first year of the emergence [14,16]. The ha-MYXV outbreaks raised additional concerns on the resilience limit of wild Iberian hare populations, due to their cumulative effect with the many insidious factors that have been responsible for the decline of both species in recent decades [17]. Despite the conservation status of the Iberian hare presently being of "Least Concern" by the International Union for Conservation of Nature (IUCN), many local populations are currently threatened as the result of a severe loss of habitat imposed by human activities, or by other infectious agents such as *Leporid gammaherpesvirus 5* (LeHV-5) that causes severe skin lesions with consequences on reproduction, or the cysticercosis, among others [18,19].

The ha-MYXV was initially detected only in Iberian hares suggesting a species-specificity for this virus. However, in mid-2020 it was also reported in diseased wild and domestic rabbits [20,21]. This finding questioned the effectiveness of current commercial vaccines against this new virus as well as the power of cross-immunity conferred by infection by the classic strains.

Furthermore, the susceptibility of the European rabbit to ha-MYXV brings additional worries towards the conservation of the species, attributed in 2019 the status "Endangered of Extinction" by the IUCN for the first time in history [22].

In the present context, the quick diagnosis of myxomatosis in wild leporids is of paramount importance for the successful management of the disease in the field, highly dependent on an early, prompt, and accurate diagnosis, given the rapid spread of the disease via direct and indirect contact between sick and healthy animals, the latter mediated by insects. For example, the laboratory confirmation of myxomatosis in hunting reserves, increases the managers' awareness to intensify active prospection and removal of sick or dead animals from the field, thus reducing the sources of infection and contamination. In some cases, the control of the disease can be accelerated by capturing and vaccinating animals.

The multiplex molecular method (mPCR) here described constitutes an essential and effective tool for (i) the quick diagnosis of myxomatosis in rabbits and hares, (ii) the differentiation of classic MYXV strains from recombinant MYXV strains (ha-MYXV), and (iii) the detection of co-infections by both virus strains while showing the capacity/fitness to be adapted to *Taqman* or *Evagreen* systems, depending on the resources available in the laboratory and technicians' preferences.

2. Results

2.1. Multiplex PCR Strategy

To optimize the diagnosis of myxomatosis, and differentiate classic MYXV from ha-MYXV strains in biological samples from European rabbit and Iberian hare, we developed a real-time quantitative multiplex PCR system (mPCR) by combining the qPCR system

previously described by [23], which detects the *m000.5L/R* duplicated gene, with two other qPCR systems developed for this study. One of those systems targets the boundaries of the 2.8 Kbp insertion within gene *m009L* (that was disrupted in the ha-MYXV genome, abrogating amplification by this system), and the other (*m060L* system) targets a small region within gene *m060L*, solely present in ha-MYXV strains. The amplification and fluorescence results expected with the different infection situations were summarized in Table 1.

Table 1. Expected results for the different targets in the mPCR. * the *m009L* system may lack the detection of some classical MYXV strains (see Table S1).

	Amplification and Fluorescence Detection			
	m000.5L/R qPCR	*m009L* qPCR	*m060L* qPCR	18S rDNA qPCR
MYXV	yes	yes/no *	no	yes
ha-MYXV	yes	no	yes	yes
Non-infected	no	no	no	yes
MYXV and ha-MYXV (Coinfection)	yes	yes/no *	yes	yes

The Table 2 contains the genomic location of the targets and sequences of the primers and probes used in the mPCR.

The 2.8Kbp insert, located around 12,335 nt from the left end of the genome (in sequence KY548791), comprises a set of six genes, namely *m060L, m061L, m064L, m065L* and M066L, some of which are truncated. These genes are transcribed to the left, showing similarity with genes *m060R, m061R, m064R, m065R* and *m066R*, present downstream in the genome between position 57,321 and 61,760 (in sequence KY548791), which are transcribed to the right.

The duplicated *m000.5L/R* gene, located in the inverted terminal repeats (TIR) at the 5′ and 3′ ends of the Myxoma virus genome, is well conserved in all classic strains as well as in ha-MYXV, conferring high sensitivity to the method developed by Duarte et al., (2014) [23], which robustness and specificity were revalidated during this study. Interestingly, the duplicated *m000.5L/R* gene is absent in the high virulent MSW strain of Californian MYXV, which also shows a deletion of 845 bp in the 3′ end of the *m009L* gene (based on the Lu sequence) [24].

An internal reference targeting the *18S rRNA* housekeeping gene was used as a reliable gene [25] to control the nucleic acid extraction process and the efficiency of each amplification reaction. The *18S rRNA* is largely used as an internal control in diagnostic tests for the detection of human and animal RNA viruses [26]. The 18S rDNA codes for the small subunit of ribosomes and is a well-preserved gene among different species within the vertebrate phylum [27] being recently validated to be used in rabbit and hare's tissues [28].

The probes' fluorophores were selected to ensure that the fluorescence of the internal control (18S) probe would be lower than that of the other probes to reduce, as much as possible, the interference with the probes directed to viral DNA. Therefore, the CY5 fluorophore was chosen for labelling the 18S probe and the minimum concentrations of the probe and primers were determined, as long as a clear reading of the results was maintained. All the quenchers used were Black Hole Quenchers (BHQ™), to avoid fluorescent quenchers (e.g., TAMRA™).

Table 2. Genomic location of the targets and sequences of the primers and probes used in the mPCR. Homology against two standard sequences (Lausanne strain (KY548791), and Tol08-18 strain (MK340973)) is marked. Underlined nucleotides represent mismatches.

qPCR System	Oligomer (Fluorophore-Quencher)	Nucleotide Sequence (5′-3′)	Position in Sequence KY548791 (Lausanne Strain)	Homologous Sequence in KY548791 (Percentage of Similarity with Primer/Probe; Gene Targeted by the PCR System)		Position in Sequence MK340973 (Tol08-18 Strain)	Homologous Sequence in MK340973 (Percentage of Similarity with Primer/Probe, Gene Targeted by the PCR System)	
				Location and Conservation of the Primers and Probes' Target Sequences in the Classic MYXV (Lausanne) and ha-MYXV (Tol08-18) Strains				
m000.5L/R (duplicated) [23]	Forward Primer	CGACGTAGATT-TATCGTATACC	558 to 537 and 161,220 to 161,241	CGACGTAGATTTATCGTATACC (100%; *m000.5L/R* gene)		564 to 543 and 163,997 to 164,018	CGACGTAGATTTATCGTATACC (100%; *m000.5L/R* gene)	
	Reverse Primer	GTCTGTCTATGT-ATTCTATCTCC	434 to 456 and 161344 to 161322	GTCTGTCTATGTATTCTATCTCC (100%; *m000.5L/R* gene)	125 bp	440 to 462 and 164,121 to 164,099	GTCTGTCTATGTATTCTATCTCC (100%; *m000.5L/R* gene)	125 bp
	Probe (Fam/BHQ1)	TCGGTCTATCCTCG-GGCAGACATAGA	483 to 508 and 161,295 to 161,270	TCGGTCTATCCTCGGGCAGACATAGA (100%; *m000.5L/R* gene)		489 to 514 and 164,072 to 164,047	TCGGTCTATCCTCGGGCAGACATAGA (100%; *m000.5L/R* gene)	
m009L	Forward Primer	TCCATTTACGATA-CACGCCGACGC	12,171 a 12,194	TCCATTTACGATACACGCCGACGC (100%; *m009L* gene)		12,147 to 12,170	TCCATTTACGATACACGCCGACGC (100%; *m009L-a* gene)	
	Reverse Primer	ACAACGTTCTATACT-GTTTAGGGGGTACG	12,316 to 12,288	ACAACGTTCTATACTGTTTAGGG-GGTACG (100%; *m009L* gene)	146 bp	15,154 to 15,127	ACAACGTTCTATACTGTTTAGGGG-TACG (97%, 1 deletion; intergenic region *m009L-a* and *m009L-b*)	No amplicon [#]
	Probe (TexasRed/BHQ2)	TACGATCTACTGAC-GAACGAATACAGTT-TAATGCC	12,254 to 12,220	TACGATCTACTGACGAACGAATAC-AGTTTAATGCC (100%; *m009L* gene)		15,093 to 15,059	TACGATCTACTGACGAACA̲ATGGAT̲ CACGGAAAGT̲ (57%; intergenic region *m009L-a* and *m009L-b*)	
m060L	Forward Primer	GATTCTTTAATCTG-GTTGAGGCAACTA	57,669 to 57,643	T̲C̲TTT̲AATCTA̲GTCGTTGC̲GAGA-AC̲A̲A̲ (48%; *m060R* gene)	No amplicon	14,723 to 14,749	GATTCTTTAATCTGGTTGAGGCA-ACTA (100%; *m060L* gene)	178 bp (*m060L*) and no amplicon (*m060R*)
						60,497 to 60,471	GT̲TTCTTTAATCTA̲GTCGT̲TGCG AGA̲A (70%; *m060R* gene)	
	Reverse Primer	GGATATTATTACGC-TCCATTATCGGAGG	57,495 to 57,522	GGATATTATTACG-CTCC̲TCTGTCGGAGG (89%; *m060R* gene)		14,900 to 14,873	GGATATTATTACGCTCCATTATCG-GAGG (100%; *m060L* gene)	
						60,320 to 60,347	GGATATTATTACGCTCCT̲CT̲GTCG-GAGG (25/28, 89%; *m060R* gene)	
	Probe (HEX-BHQ1)	CTGATAAGTACC-CCTTATCTACAAAA-ACGGGTG	57,639 to 57,607	CTGC̲C̲AAA̲TATCCCTTATCC̲ACGC̲A-AA̲TGGGA̲G (73%; *m060R* gene)		14,756 to 14,788	CTGATAAGTACCCCTTATCTACAAA-AACGGGTG (100%; *m060L* gene)	
						60,471 to 60,320 *	(No significant similarity; *m060R* gene)	

* Region amplified by the primers, [#] No signal due to the lack of amplification due to the relative position of the primers 2,946 bp apart; underlined nucleotides indicate no homology between primer sequence and target sequence.

2.2. Specificity of the Primers and Probes

The specificity of the three PCR systems (m000.5L/R, m009L and m060L) was first determined in silico, against the representative sequences available in the NCBI database, by using the BlastN analysis service from NCBI. Despite the evaluation of the specificity based only on the number and position of oligomers' mismatches can be misleading [29], in silico validation of polymerase chain reaction primers and probes is a common procedure [30] to predict the functionality of the amplification reactions.

Therefore, to further confirm the specificity of these three systems, the nine oligomers were tested in silico (September, 2021) for potential annealing with the genomes of rabbit and hare pathogens with very relaxed temperature conditions (55 °C), showing all matching sites of primer binding, a length of 60 bp to 3000 bp for the amplicon and allowing mismatches in 5 nucleotides of the 3′ end), looking for all possible connection points using the software FASTPCR 6.7 (PrimerDigital, 2020). The pathogens investigated included *Rabbit fibroma virus* (NC_001266), *Goatpox virus* (NC_004003), *Murmansk poxvirus* (NC_03546), *Squirrel poxvirus* (NC_022563), *Yoka poxvirus* (NC_015960), *Murmansk poxvirus* (MF001304), *Teiidae poxvirus* (MT712273), *Cheloniid poxvirus 1* (MT799800), *Bibersteinia trehalosi* (NZ_CP006954), *Chlamydophila abortus* (CR848038), *Coxiella burnetii* (CP040059), *Cryptosporidium parvum* (CM000436), *Escherichia coli* (AE014075.1), *Encephalitozoon cuniculi* (LFTZ01000003), *Enterococcus faecalis* (CP045918), *Francisella tularensis* (CP025778), *Klebsiella pneumoniae* (FO203501), *Leptospira interrogans* (CP039256), *Mannheimia haemolytica* (CP006957), *Pasteurella multocida* (CP031552), *Salmonella enterica* (CP003278), *Serratia* sp. (CP025085), *Staphylococcus aureus* (AP017922), *Staphylococcus* epidermidis (CP043847) and *Toxoplasma gondii* (U87145). Despite viral RNA not being amplified by this multiplex PCR, a few RNA genomes were also evaluated given the possibility, in the future, of including these primers on a wider multiplex PCR System for RNA and DNA viruses. The three RNA viruses evaluated included *hare lagovirus* (KR230102.2 and MK138384), RHDV (MF421574) and EBHSV (MK440616).

The putative results of the quadruplex system deduced from the BLAST analysis are shown in Table S1. From a total of 85 genomes, including all the representative strains of myxoma virus available in the NCBI database since 1949 (until September 2021), only one strain was not detected by this system, namely the California/San Francisco MSW strain, isolated in 1950 (KF148065). So far, the recent ha-MYXV strains are unique being detected by the M060L system.

The low variability of the *m000.5L/R* gene, and its duplication at the opposite ends of the genome, makes it an excellent candidate to assure that, if present, all MYXV strains are detected. On the contrary, the *m009L* gene is prone to mutations, deletions and additions, making it ideal to detect the current European MYXV strains and to evaluate genetic variability and virus evolution.

None of the pathogens' sequences and samples listed in this and 4.4.1 subsections, analysed in silico and in vitro, respectively, were detected by any of the three methods. Likewise, in silico analysis of the heterologous vaccines (Shope Fibroma Virus vaccines, namely Mixohipra FSA, Mixovacina and Lyomyxovax) showed that none of these vaccines was detected by any of the three systems. In the case of homologous vaccines (Myxo-RHD Plus, Dervaximyxo, Mixohipra-H, POX-LAP and Dercunimix SG33), none was detected by the *m060L* system as expected, all vaccines were detected by the *m000.5L/R* system, and only Dervaximyxo and Dercunimix vaccines were detected by the *m009L* system (Table S1).

Most homologous vaccines underwent genetic modifications in the *m009L* gene during cell culture passages, or as a result of natural changes or genetic manipulation of the virus strains, to attenuate the virus. In fact, during this study we sequenced a few commercial vaccines, confirming this variability (not shown for confidentiality reasons). Regarding the European classic field strains, except for one strain from Germany (1985, KP723387), one strain from Spain (1995, EU552530) and two strains from England (2009, KY548812 and 2011, KY548813), the target regions of the *m009L* primers and probe are fully conserved in all the classic strains which sequences are available in the NCBI (Table S1). On the contrary, in the ha-MYXV strains the *m009L* region is interrupted by the insertion of a

2.8 kbp additional sequence, separating the two target sequences of the *m009L* primers by around 3.0 kb, and interrupting the probe-target sequence, therefore hampering annealing. Consequently, the *m009L* method does not detect these strains, leading to negative results. The positivity in the *m000.5L/R* system excludes false-negative results for myxomatosis.

2.3. Limit of Detection and Sensitivity of the Uniplex Systems

The minimum copy number of MYXV DNA detected by the *m000.5L/R* uniplex PCR was 3 copies, following the value previously described (2.6 copies) by Duarte et al. [23]. Both the *m060L* and *m009L* systems demonstrated to be able of detecting as few as 3 copies of viral DNA. In the uniplex modality, the amplification efficiency for the *m060L*, *m009L* and *18S rRNA* targets was above 98% (Figure 1).
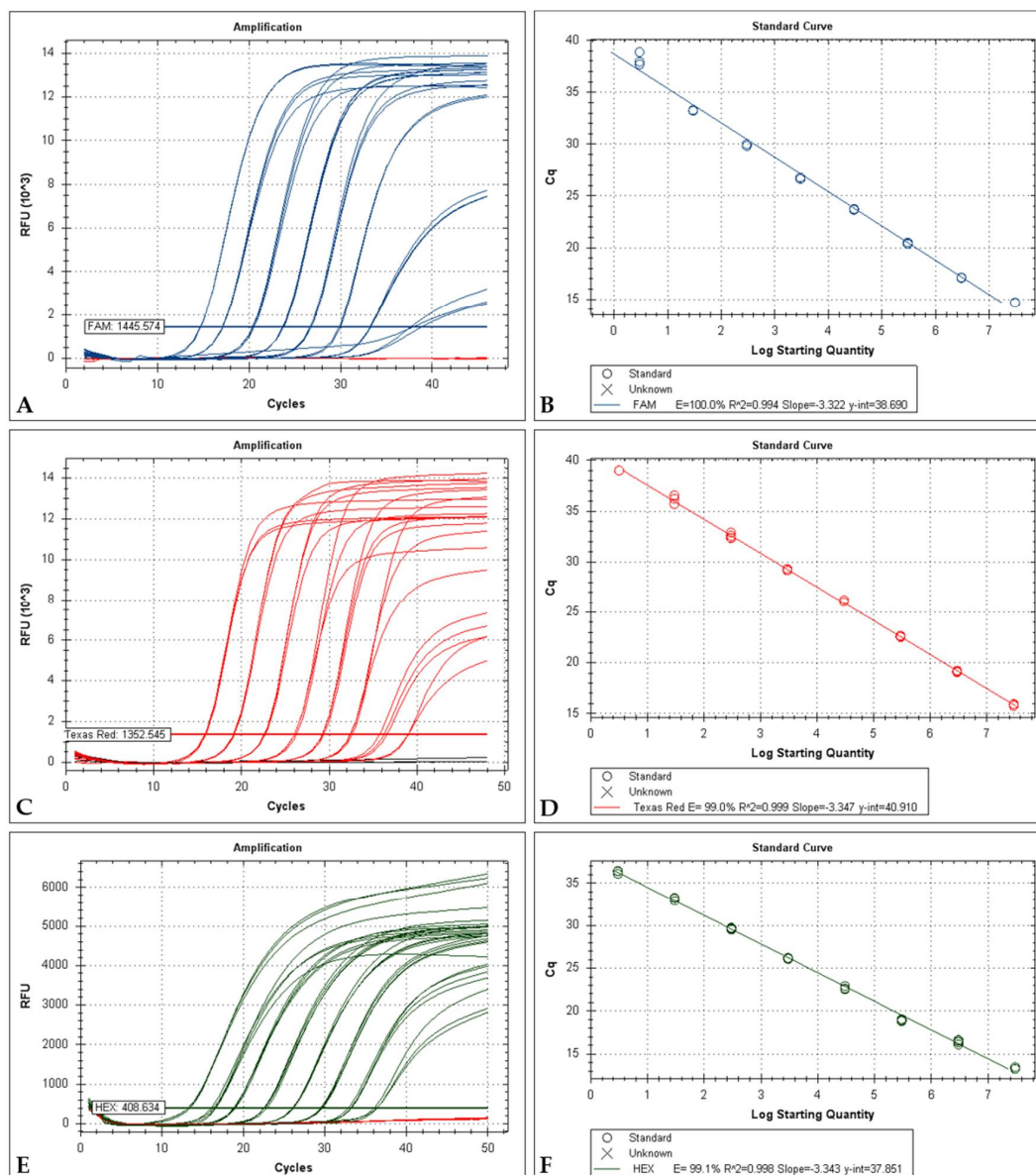
Figure 1. (**A**) Serial dilution from 3.0×10^7 to 3.0×10^0 copies of *m000.5L/R* per reaction. Red lines correspond to negative controls (3.0×10^{-1} copies). (**B**) Standard curve for the *m000.5L/R* qPCR, the log starting quantity ranged from 3.0×10^7 to 3.0×10^0 copies in the final reaction. (**C**) Serial dilution from 3.0×10^7 to 3.0×10^0 copies of *m060L* per reaction. Black lines correspond to negative controls (3.0×10^{-1} copies) (**D**) Standard curve for the *m060L* qPCR, the log starting quantity ranged from 3.0×10^7 to 3.0×10^0 copies in the final reaction. (**E**) Serial dilution from 3.0×10^7 to 3.0×10^0 copies of *m009L* per reaction. Red lines correspond to negative controls ($3.0 10^{-1}$ copies). (**F**) Standard curve for the *m009L* qPCR, the log starting quantity ranged from 3.0×10^7 to 3.0×10^0 copies in the final reaction.

Typical standard curve amplification plots and linear regression analyses were found for the three singleplex (Figure 1). Excellent linearity was observed over 8 orders of magnitude, from 3×10^7 to 3×10^0 copies. The regression analyses for these intervals yielded R^2 (correlation coefficients) of 0.99. The slopes around 3.3 revealed high qPCR efficiency (Figure 1B,D,E).

2.4. Determination of the mPCR Optimal Conditions and 3D Plot Analysis for the Quadruplex System

The initial optimization of the primers and probe concentrations in the mPCR modality revealed an expected cross-effect between the different systems concerning the concentrations of the primers. Figure 2 illustrates the optimization of primer concentration of each system to obtain balance in the amplification efficiency of the three systems and Cycle quantification.
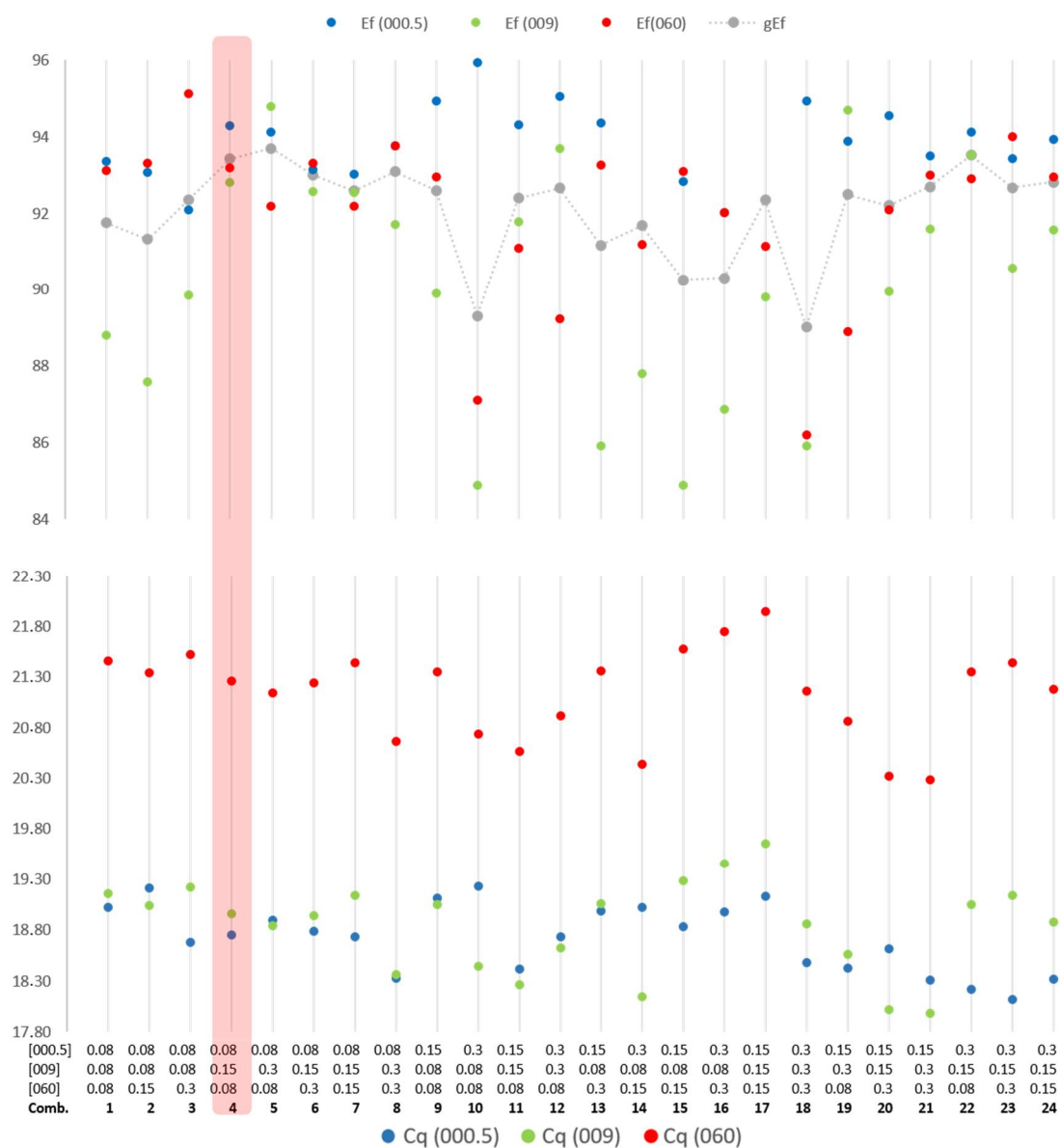
Figure 2. Graphical presentation of mPCR performance using different primers concentrations ([m000.5], [m009], [m060]) The grey dots are joined together (therefore not representing a trend line) to illustrate the variation in average efficiency.

All combinations of variable primers and probes concentrations that generated amplification efficiencies below 90% for the three systems, or at least for one, were excluded. This

was the case for combinations number 1, 2, 3, 10, 13, 14, 15, 16, 17, 18 and 20, illustrated in Figure 2. As shown in Figure 2, combination 4 was chosen based on the good efficiency, adequate Cq values, and sigmoidal curve shapes (not visible in the figure). This combination was then extensively tested and optimized for small variations in the concentrations of primers and probes (results not shown) to balance the fluorescence between the four systems (avoiding fluorescence saturation).

The selected conditions based on the 3D Plot analysis were annealing temperature of 60 °C, the Mg^{2+} concentration recommend by the kit manufacture (2.5 mM) and the dNTP concentration kept to the minimum (0.2 mM).

2.5. Primer and Probes—Recommended Concentrations and mPCR Protocol

The final concentrations for the primers and probes and the optimized protocols for the mPCR are shown in Tables 3 and 4, respectively. However, these conditions should be optimized for other enzymes and devices.

Table 3. Optimized primers and probes' concentrations for field samples in the mPCR.

	Concentrations in the Amplification Reaction (µM)		
Gene	Primer Fw	Primer Rv	Probe
m000.5L/R	0.08	0.08	0.07
m009L	0.12	0.12	0.14
m060L	0.08	0.08	0.1
18S rRNA	0.08	0.08	0.1

Table 4. Optimized protocols for quadruplex PCR with Taqman probes, and triplex PCR using EvaGreen.

Amplification Protocols for mPCR	
Taqman System	Evagreen System
Step 1: 95 °C for 4:00	
Step 2: 94 °C for 0:30	
Step 3: 60 °C for 0:30	Step 1: 98 °C for 2:00
Step 4: 72 °C for 0:30	Step 2: 98 °C for 0:05
Go to step 2: Repeat 2×	Step 3: 60 °C for 0:15
Step 5: 94 °C for 0:30	Go to step 2: Repeat 39×
Step 6: 60 °C for 0:30	Melt curve: 65–95 °C (0.3 °C increment,
Plate read	5 s/step)
Step 7: 72 °C for 0:30	
Go to step 5 Repeat 36×	

2.6. Detection Limit and Sensitivity of the Optimized mPCR

The mPCR system was able to detect as few as 9 molecules of DNA per 50 µL of PCR reaction of *m000.5L/R*, *m060L* and *m009L* genes. The ten-fold serial dilutions standard curves generated by the quadruplex system corresponding to 9.0×10^7 to 9.0×10^1 copies per reaction are shown in Figure 3, resulting from an average of 3 standard curves for each target. The dilutions of 9.0×10^0 were excluded from the standard curve analysis because of the scale, curve shape and high coefficient of variation.
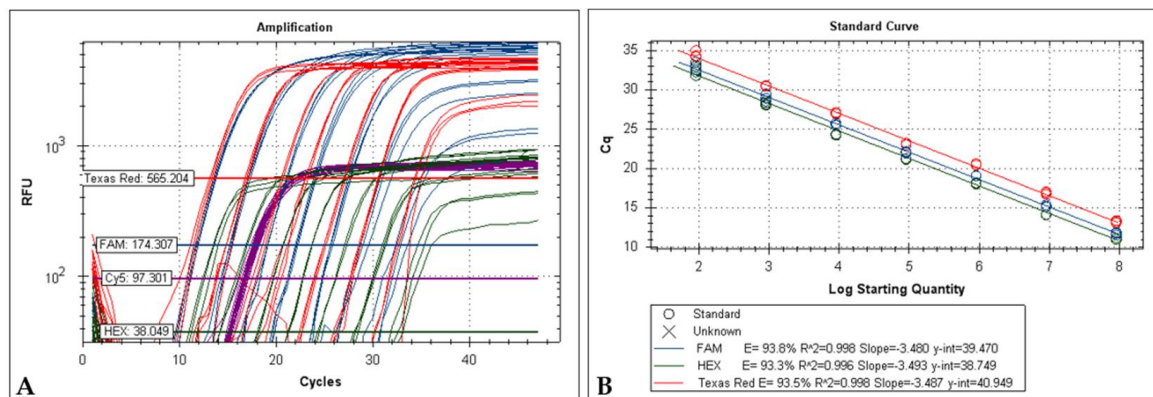
Figure 3. (**A**) Serial dilution from 9.0×10^7 to 9.0×10^1 copies of each plasmid (pM000.5L/R, pM009L and pM060L) and a constant 3×10^6 copies of p18S per reaction. Red curves (TexRed) correspond to *m060L* system, blue curves (FAM) corresponding to *m000.5L/R* system, green curves (HEX) corresponding to *m009L* system and purple curves (CY5) correspond to 18S system. (**B**) Standard curve for the quadruplex qPCR. The reaction copies ranged from 9.0×10^7 to 9.0×10^1 copies in the final reaction.

2.7. Intra and Inter-Assay Variation

To evaluate intra-assay variability or repeatability of the qPCR quadruplex, the coefficient of variation (CV%) was calculated using the Cq values from the quadruplicates of each dilution. The maximum CV determined was 3.65%, close to the ideal maximum value of 3.5%. To access plate-to-plate consistency, the inter-assay variation or reproducibility, the %CV was calculated using the Cq values from duplicates in two different runs. The inter-assay variability demonstrated the good reproducibility performance of the multiplex with Cq values generating CVs under 3.52%. The Cq mean standard deviation and CV% values obtained with the mPCR are summarized in Table 5.

Table 5. Repeatability and reproducibility of the mPCR.

Target	N. of Copies/Reaction	Intra-Assay (Quadruplicates)			Inter-Assay (2 Independent Assay with Duplicates)		
		Mean	SD	CV%	Mean	SD	CV%
pM000.5L/R	9.0×10^7	11.74	0.17	1.47	-	-	-
	9.0×10^6	15.23	0.13	0.83	15.13	0.32	2.12
	9.0×10^5	19.09	0.02	0.10	-	-	-
	9.0×10^4	22.08	0.10	0.44	22.68	0.45	1.98
	9.0×10^3	25.62	0.14	0.54	-	-	-
	9.0×10^2	29.00	0.45	1.55	29.53	0.27	0.91
	9.0×10^1	32.86	0.27	0.82	-	-	-
	9.0×10^0	36.04	1.27	3.52	36.24	1.21	3.34
pM060L	9.0×10^7	13.25	0.11	0.83	-	-	-
	9.0×10^6	16.89	0.17	0.99	16.72	0.25	1.50
	9.0×10^5	20.55	0.14	0.69	-	-	-
	9.0×10^4	23.09	0.12	0.53	23.32	0.27	1.16
	9.0×10^3	27.04	0.08	0.31	-	-	-
	9.0×10^2	30.05	0.06	0.20	30.82	0.32	1.04
	9.0×10^1	34.52	0.42	1.21	-	-	-
	9.0×10^0	37.91	1.29	3.40	37.52	1.32	3.52
pM009L	9.0×10^7	11.32	0.41	3.65	-	-	-
	9.0×10^6	14.15	0.35	2.47	14.74	0.36	2.44
	9.0×10^5	18.10	0.07	0.40	-	-	-
	9.0×10^4	21.21	0.03	0.16	21.53	0.38	1.76
	9.0×10^3	24.32	0.10	0.40	-	-	-
	9.0×10^2	28.21	0.17	0.59	29.14	0.43	1.48
	9.0×10^1	32.47	0.63	1.92	-	-	-
	9.0×10^0	35.91	0.92	2.56	35.73	1.13	3.16

2.8. Eva Green-Based Multiplex Real-Time PCR (EG-mPCR)

The mPCR was adapted to EvaGreen to allow its use without the need of purchasing expensive Taqman probes. In singleplex amplification, three distinct peaks (Figure 4A) were observed corresponding to good dissociation curves (Figure 4B). Figure 4A shows the Evagreen amplification peaks in the Evagreen systems of two out of the eight dilutions tested of plasmids pM060L, pM000.5 L/R and pM009L.
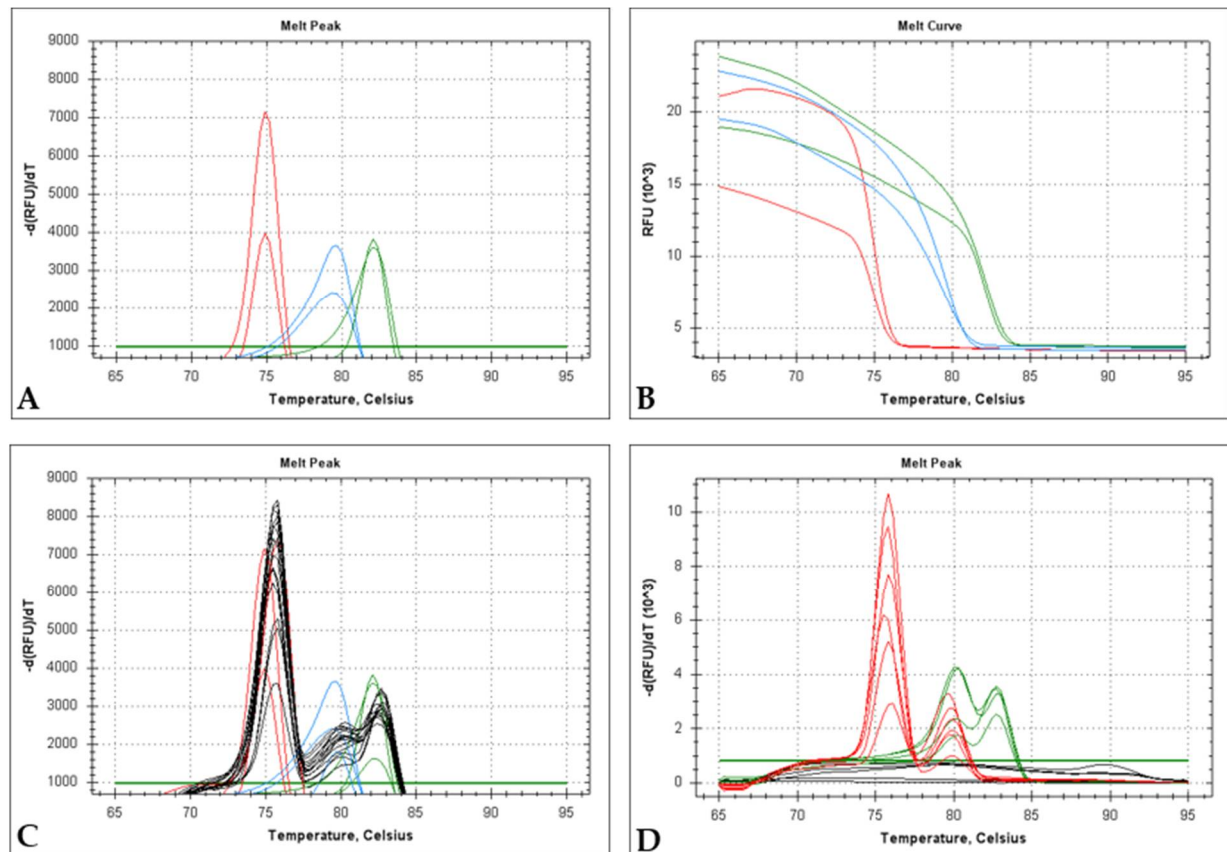
Figure 4. (**A**) Melting peaks of EvaGreen-PCR in singleplex reactions. Red lines, blue lines and green lines correspond to *m060L*, *m000.5L/R* and *m009L* systems, respectively. (**B**) Dissociation curves from (**A**). (**C**) Melting peaks analysis for the three systems described in (**A,B**), superimposed by the respective triplex analysis (three plasmids in the reaction, black lines), with the correspondent three peaks. (**D**) Melting peaks analysis for field samples using the EvaGreen-mPCR, where red lines represent samples positive to ha-MYXV and green lines samples positive for classic MYXV strains.

Triplex analysis using eight tenfold dilutions of the same plasmids generated three distinct peaks (Figure 4C, black lines), homologous to those observed in the singleplex reactions (Figure 4A,C).

When field samples ha-MYXV-positive were tested, two peaks were observed corresponding to the *m060L* (left red peak) and *m000.5L/R* (right red peak) genes specific amplification. Two peaks were also detected with classic MYXV-positive samples, corresponding to the amplification of the *m000.5 L/R* (left green peak) and *m009L* (right green peak) genes. The melting temperatures of each one of the three amplicons proved to be highly stable and differentiated, regardless of the modality of the qPCR used (Table 6).

Table 6. Comparison of the melting temperatures obtained for the singleplex and multiplexes.

	In Uniplex Format			In Triplex Format		
PCR system	*m000.5L/R*	*m060L*	*m009L*	*m000.5L/R*	*m060L*	*m009L*
Mean ± SD	80.07 ± 0.08	75.58 ± 0.10	82.54 ± 0.28	80.11 ± 0.24	75.43 ± 0.18	82.34 ± 0.31

The The melting temperature peak for each system was determined by the mean ±SD of four duplicates replicated in two different runs.

2.9. Field Sample Analysis

To validate the developed mPCR system for routine diagnosis, the multiplex system protocol with Taqman probes was used to test field samples from National Institute for Agricultural and Veterinary Research—INIAV-biobank, previously classified as positive or negative for MYXV and ha-MYXV using the clinical and necropsy data, a qPCR recommended by the OIE Terrestrial Manual [31] and a conventional PCR previously published [12]. A sample is considered ha-MYXV-positive (Figure 5A) in the mPCR when the qPCRs targeting the *m000.5L/R*, *m060L* and *18S rRNA* genes generate fluorescence signals with Cq values below 38.0, and the *m009L* system does not amplify DNA from the sample (Table 2). On the other hand, a sample is considered classic MYXV-positive (Figure 5B) when the qPCR targeting the *m000.5L/R*, *m009L* and *18S rRNA* genes generate fluorescence signals with Cq values below 38.0, and the M060L system does not amplify DNA from the sample been tested (Table 2). Classic MYXV and/or ha-MYXV positive samples generate exponential fluorescence in channels CY5 (18S rDNA amplification, Cq values < 38.0) and FAM (*m000.5 L/R* amplification). Using the mPCR system developed, we were able to confirm the results previously obtained regarding 59 out of the 60 samples tested (98.33%) (Table 7). For one classic MYXV-positive sample, tested in duplicate, no amplification was obtained with the m009L system in the quadruplex PCR, probably due to the lower efficiency of the system compared to the singleplex. Nevertheless, this sample showed a positive result in the *m000.5 L/R* system (Cq 35.2, considered as very low viral load), confirming the presence of MYXV-DNA in the sample. When in uniplex, amplification of this sample with *m009L* was successful (Cq 37.1). None of the field samples analysed so far by our team was simultaneously positive for both viruses, indicating that co-infection by MYXV and ha-MYXV is not a common event.
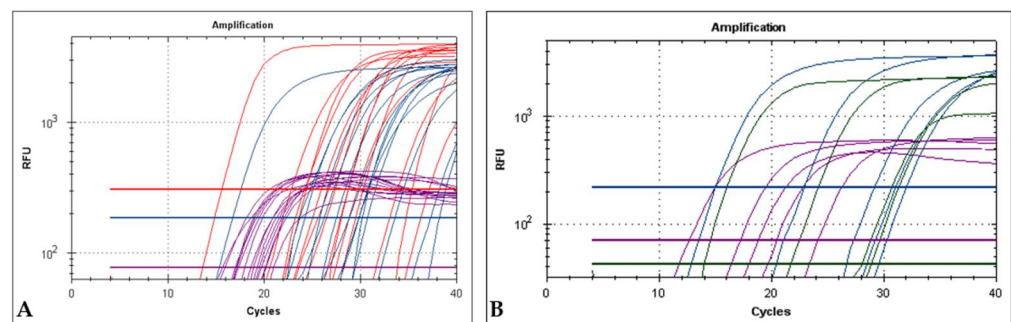
Figure 5. (**A**) Results in field samples positive to ha-MYXV (red sigmoid curves: *m060L*, Texas Red channel; blue sigmoid curves: *m000.5L/R*, FAM channel; green flat lines: m009L, HEX channel, not visible due to scale). (**B**) Results with field samples positive for classic MYXV (blue curves: *m000.5L/R*, FAM channel; green curves: *m009L*, HEX channel, red flat lines: *m060L*, Texas Red channel, not visible due to scale). All samples were positive for 18S rDNA amplification (CY5, purple curves).

Table 7. Comparison of the qualitative results obtained with the *m000.5L/R* singleplex and quadruplex.

	Singleplex (*m000.5L/R*)	Quadruplex (*m000.5 L/R*, 18S rDNA, *m060L* or *m009L*)
True positive	41 (24 ha-MYXV positive and 17 classic MYXV positive)	40 *
True negative	19	19
False positive	0	0
False negative	0	1 *

* One sample in the quadruplex analysis was positive for *m000.5L/R* (Cq 37.2) and *18S rRNA*, negative to *m009L* and *m060L*. Results from the singleplex: positive for *m009L*, negative for *m060L*).

The lower fluorescence of the 18S PCR system compared with the fluorescence reached with the *m060L*, *m000.5 L/R*, and *m009L* qPCRs (Figure 5) was imposed by limiting the primers and probe concentrations. Despite this, the CY5 low fluorescence does not compromise the validity of the extraction and PCR, and minimizes the interference between channels and fluorescence saturation.

3. Discussions

Wild leporids are an iconic species of the Iberian Peninsula, where they have tremendous ecological, cultural, and economic importance. Myxomatosis has been a severe menace for the wild rabbit since it emerged on the Iberia Peninsula in 1980. In 2018, the disease emerged for the first time in the Iberian hare [15,32] causing high mortality in the wild populations and aggravating the decrease of this species in both Portugal and Spain [19]. In Portugal, myxomatosis accounted for 27.8% of the mortality of wild rabbits verified in the field between mid-2017 and late 2020 (data from the Action Plan for the control of RHDV2 in wild rabbits, dispatch 4757/17 of May 31) and for 88% of the mortality of Iberian hares (after the emergence of ha-MYXV in October 2018), accelerating the reduction of the wild populations of both species that have been observed in recent decades, also, as a result of land-use changes.

The impact of myxomatosis in the wild leporids populations makes the existence of a rapid, less laborious and low-cost tool for laboratory diagnosis of the disease and virus discrimination/characterization in myxomatosis outbreaks in these species imperative. Here we describe the first real-time PCR method to detect ha-MYXV strains that simultaneously enables investigating the presence of classic MYXV strains and putative co-infections. The three PCR systems that are integrated into the mPCR are validated by the amplification of internal control (*18S rRNA* gene), previously demonstrated to be a strong and stable housekeeping gene (Abade dos Santos et al., 2021). The sensitivity and specificity of these PCR systems were 100% when samples previously classified as positive or negative were screened by the *m000.5L/R*-based system [23] and further classified as recombinant/classic MYXV by the system developed by [12].

When the four systems were used together in the quadruplex, the mPCR proved to be robust, highly sensitive and highly specific. The technique has the advantage of being applied using two different PCR formats, according to the laboratory preferences, namely by hydrolysis of a Taqman probe or green fluorescent nucleic acid dying (EvaGreen). Theoretically, the cycle quantification values in the multiplex (shown in Figure 3 and Table 5) and uniplex assays (shown in Figure 1) should be superimposable. However, during the in vitro assays, the efficiency interference and the fluorescence crossover between probes deviate from these values, despite still being relatively close.

The *m060L* system is directed to a gene of the ha-MYXV 2.8 kbp insert that is homologous to *m060R* gene, located further downstream in both classic MYXV and ha-MYXV genomes. However, as the primers and probes target sequences within the *m060R* gene, present a high number of mismatches (>9) in classic MYXV strains (Lausanne strain, KY548791) no amplification occurs under the conditions used. Even under very permissive conditions (such as an annealing temperature of 56 °C, and 40 cycles) neither exponential amplification was detected in the real-time PCR nor amplicon was synthesized using the primers in a conventional PCR format. We demonstrated the absence of amplification of the *m060R* gene with the oligomers of the *m060L* system in 20 samples from classic MYXV infected rabbits. The mPCR method has two levels of internal validation, through the *m000.5L/R* and *18S rRNA* systems. Amplification of the 18S rDNA target [28] validates the quality of tissue, the extraction and amplification efficiency, while amplification of the duplicated *m000.5L/R* target, allows for the presumptive initial diagnosis of the infection. Furthermore, the amplification of target *m000.5L/R* allows the exclusion of potential recombination in genes *m009L* and *m060L* that could lead to false-negative results. m009L negative results could also occur due to mutations in this gene, as verified in silico for some strains, namely Australian strains and vaccine strains. Only the Dervaximyxo and

Dercunimix were detected by in silico analysis using the *m009L* system (Table S1). Despite the gene *m000.5L/R* being duplicated, the copy number must be similar to the other target genes, meaning that the Cq value should be similar (Cq value of *m000.5L/R* similar to Cq value of *m009L* in animals infected with classic MYXV and Cq value of *m000.5L/R* similar to Cq of *m060L* in animals infected with classic MYXV), apart from the difference in their efficiencies and threshold line (Table 5).

A Cq value in the 18S rDNA channel $\geq$ 30.0 could mean problems with the original sample quality (e.g., degradation or over dilution), in the extraction or during the PCR (e.g., inhibitors). Simultaneous amplification of *m000.5L/R*, *18S rRNA*, *m009L* and *m060L* could mean co-infection with classic and recombinant MYXV.

Although no leporids were found co-infected by classic and recombinant strains of MYXV, this situation was simulated in three different ways: (i) with samples containing the four plasmids (pM000.5, pM060, pM009 and p18S), (ii) with nucleic acids extracted from a mixture of eyelids from rabbits infected with the two viruses and (iii) with a mixture of DNA obtained separately from the two strains. In all three situations, simultaneous amplification of MYXV and ha-MYXV DNA took place either using the Taqman or the Evagreen system.

m009L is a frequently mutated gene as shown by the BLAST analysis (Table S1), with some geographic related variations, such as single deletions or substitutions [33] in the Australian strains. In some strains, these genetic mutations prevent the amplification by the *m009L* system (Table S1). The *m009L* gene is also a target of larger deletions, for example in strain 6918 [11], despite not affecting the m009L detection. Since *m009L* was the target of the main genetic change found in the ha-MYXV strains, it was chosen for the mPCR, functioning as a monitor of this specific genetic region. In fact, of all the representative sequences currently available in the NCBI database, all classic MYXV Europe strains (except the four strains referred to in Section 2.2) would be detected in the m009L system (Table S1). On the other hand, using the *m000.5L/R* system in parallel, the detection of the virus is guaranteed even in the absence of virus detection by the *m009L* or *m060L* systems due to mutations.

The systemic distribution of the MYXV during infection allows the diagnosis to be performed using different organs, although with variable Cq values (e.g., lower in the skin with lesions, and higher in spleen and liver). Additionally, given the need to perform diagnosis in wild animals, often undergoing some degree of degradation and autolysis, we recommend that samples with Cq values over 38.0 but with a sigmoid shape, be further investigated by repeating the analysis using the duplex system (18S and target amplicon) before being considered negative.

To our best knowledge, this is the first real-time PCR method for ha-MYXV diagnosis in both rabbits and hares and proved to be a robust technique for differentiation between classic and natural recombinant strains using two alternative systems, according to preference.

4. Material and Methods

4.1. Biologic Samples and DNA Extraction

A total of 60 samples from the biobank of the National Reference Laboratory for Animal Diseases (INIAV I.P.), collected between 2017 and 2021 from wild leporids all over the Portuguese national territory were used in this study. The samples had been previously tested for myxomatosis by molecular methods and submitted to the anatomopathological examination and in vitro virus propagation [16,23,34].

For nucleic acid extraction, fresh or frozen samples of tissues from of the eyelids and liver/spleen from ha-MYXV or classic MYXV positive leporids were homogenized at 20% (w/v) with phosphate-buffered saline (PBS) and clarified at $3000\times g$ for 5 min. Total DNA and RNA were extracted from 200 μL of the clarified supernatants using the MagAttract 96 cador Pathogen Kit (Qiagen, Hilden, Germany) in a BioSprint 96 nucleic acid extractor

(Qiagen, Hilden, Germany), according to the manufacturer's protocol. Nucleic acids were preserved at −20 °C until use.

4.2. Primers and Probes Design

During this study, a Taqman probe system was designed to specifically detect ha-MYXV strains based on the amplification of a 178 bp-long genomic region corresponding to part of gene *m060L*, only present in the 2.8 Kbp insert.

For the detection of classic MYXV strains, a Taqman probe system was designed based on the amplification of an internal region of gene *m009L*. This 146 bp-long region is only present in the genome of classic myxoma virus strains since it encompasses the zone disrupted by the 2.8 Kbp insertion in ha-MYXV strains

Primers and probes were designed with the PCR Primer Design software (Eurofins Genomics, Ebersberg, Germany) and manually optimized, based on the alignment of six recombinant MYXV strains' sequences obtained from Iberian hares (MK340973 and MK836424), domestic rabbits (MT920563 and MT920564) and wild rabbits (MT940239 and MT940240). The nucleotide sequences of the nine oligomers used are described in Table 2.

The four systems (*m000.5L/R*, *m009L*, *m060L* and *18S rRNA*) were manually optimized to allow the use of the same annealing temperature in the multiplex assay and to generate amplicons with differentiable melting temperatures necessary for melting curve analysis.

4.3. Cloning and Sequencing of the qPCR Amplicons

Amplicons were obtained by conventional PCR with the four sets of primers mentioned above, using DNA extracted from a ha-MYXV positive hare (for *m060L* amplification), an MYXV-positive rabbit (for *m000.5L/R* and *m009L* amplification) and a healthy wild rabbit (for *18S rRNA* amplification). Amplifications were carried out using the FastStart TaqMan Probe Master Kit (Roche, Roche Diagnostics GmbH, Manheim, Germany), in a 25 μL final reaction volume using 12.5 μL of 2× buffer, 0.5 μL of forward primer (0.2 μM final concentration), 0.5 μL of reverse primer (0.2 μM final concentration), 5 μL of DNA template and 6.5 μL of RNase/DNase free water. Denaturation and extension conditions were carried out according to the instructions in the PCR kit, using an annealing temperature of 60 °C. The qPCR reactions were conducted in a Bio-Rad's CFX96 Dx Real-Time PCR equipment.

The size of the amplicons was confirmed by agarose gel electrophoresis, and the reaction products were directly cloned into the pCR2.1 TA vector (Invitrogen Corporation, San Diego, CA, USA) accordingly with the manufacture's recommendations. Five recombinant plasmids containing the inserts (pM000.5 L/R, pM060L, pM009L, p18S(hare), p18S(rabbit)) were purified from *Escherichia coli* recombinants grown in Luria Broth medium, at 37 °C with 220 rotations per minute, using the NZYMiniprep Kit (NZYTech, Lisbon, Portugal). Both strands of the inserts were cycle sequenced using the M13F and M13R primers and a 3130 Genetic Analyser (Applied Biosystems, Foster City, CA, USA). Sequences were assembled and edited using the BioEdit 7.2 software (Bioedit, Manchester, UK).

4.4. Detection Limit, Sensibility, Specificity, Repeatability and Reproducibility
4.4.1. Specificity: In Vitro Analysis

Each one of the three qPCR was optimised individually. While the m000.5L/R system efficiency, sensitivity and specificity were previously demonstrated (Duarte et al., 2014), the specificity of the primers and probes for the *m060L* and *m009L* qPCR systems, were tested in vitro against total nucleic acids extracted from animal tissues infected with viruses including an avian poxvirus (*Fowlpox virus*) and leporid virus (*rabbit haemorrhagic disease virus*, *Leporid gammaherpesvirus 5*). Healthy rabbit and hare tissues, DNA from isolated from bacteria (*E. coli*, *P. multocida*, and *S. aureus*), and parasites (*Cysticercus pisiformis*, *Eimeria stiedae*, *Eimeria* spp. and *Passalurus ambiguous*), available at the National Reference Laboratory for Animal Health (INIAV, I.P, Oeiras, Portugal) were also tested. In all qPCR reactions performed, 20 ng of DNA template were used. Amplifications were performed

using duplex systems which included the 18S internal control and one specific target at a time. A relaxed annealing temperature of 55 °C was used to decrease the specificity.

Additionally, all the available myxomatosis (with homologous and heterologous virus) vaccines were tested by in silico and/or in vitro analysis (Table S1). The conditions for in silico analysis were described above (Section 2.2).

4.4.2. Detection Limit, Sensibility, Repeatability and Reproducibility

The DNA concentration of the purified recombinant plasmids was determined by A260 measurement (Qubit 4 Fluorimeter by Invitrogen) and the copy number calculated based on the plasmids molecular weight, where 3929 bp corresponds to the plasmid plus the insert (amplicon of interest).

After plasmid purification, the DNA concentration and purity of each plasmid (for each system) was distinct from the others. From each purified DNA, an initial dilution corresponding to 6×10^6 copies/µL (corresponding to 3.0×10^7 copies of DNA template in 5 µL, the volume added to the PCR reactions) was prepared and from these 10-fold serial dilutions were made. All the dilutions were performed using Nuclease-Free Water, for Molecular Biology (Sigma-Alrich, St. Louis, Missouri, USA). For each system, the standard curves were constructed using three replicates per dilution by plotting the cycle threshold (Cq) values against the logarithm of the DNA copy number. The absolute copy number of plasmid concerning the starting amount of DNA was calculated accordingly to the formula: Number of copies detected = amount of DNA (g) $\times$ 6.022 $\times 10^{23}$/fragment (bp) $\times$ 650.

The four singleplex PCRs were performed with the Multiplex PCR NZYTaq 2$\times$ Colourless Master Mix (NZYTech, Lisbon, Portugal) in a final volume of 25 µL, containing 12.5 µL of 2$\times$ buffer, 0.5 µL of forward primer (0.2 µM final concentration), 0.5 µL of reverse primer (0.2 µM final concentration), 0.5 µL of probe (0.2 µM final concentration), 5 µL of DNA template and 6.5 µL of RNase/DNase free water.

4.5. Optimization of the Multiplex Real-Time PCR Assay (mPCR)

After optimization of the four singleplex qPCR (*m000.5L/R*, *m060L*, *m009L* and *18S rRNA*), the final concentration of each of the eight primers and four probes combined was optimized in the mPCR to avoid cross-inhibition or interferences between the different fluorescence signals.

While maintaining the 18S primers and probe concentrations to a minimum (Table 3), their effect in the efficiency of amplification (comparing assays with or without the 18S system), average efficiency and Cycle threshold for the other three targets were evaluated by varying the concentrations of the primers and probes (Figure 2). The efficiency was estimated using three replicates (the mean value is shown the Figure 2) for three different concentrations of each target (9×10^5, 9×10^4 and 9×10^3 copies per reaction), using the respective recombinant plasmids. The p18S plasmid was kept stable at 3×10^6 copies per reaction. The Cq values shown in Figure 2 correspond to three replicates (average value presented) using 9×10^5 copies of each plasmid (pM060, pM009, pM000.5) and 3×10^6 copies of p18S.

The response surface method (RSM) was used to evaluate the effects of interactions between variables (primers, probe and Mg^{2+} concentration). For this analysis, a fixed number of copies (3×10^5) of the recombinant plasmid was used. The conditions testes were dNTP concentration (0.2, 0.3, 0.4 mM), MgCl$_2$ concentration (2.5, 3, 4, 5 and 6 mM), and annealing temperature (52, 52.7, 54, 55.9, 58.4, 60.3, 61.4, 62 °C). The optimal values were selected to balance the different intrinsic fluorescence of the fluorophores and the efficiencies of the different systems so that a balanced amplification and near fluorescence of the various targets occurs.

The final concentrations were still submitted to final optimizations to minimize the fluorescence differences between the probes and the different efficiency of the systems, in which the denaturation, annealing and extension times of the Taqman and Evagreen

systems were also evaluated. All mPCR reactions were prepared in a final volume of 50 μL. The final conditions are shown in Tables 3 and 4.

4.6. Field Samples Analysis

The sensitivity and specificity of the method were evaluated with 60 field samples obtained between 2017 and 2021 from the INIAV biobank, comprising 10 hare and 9 rabbit samples that had tested negative to MYXV and ha-MYXV, 17 rabbit samples that tested positive for MYXV, and 20 hare and 4 rabbit samples that tested positive to ha-MYXV. All samples were analysed in duplicate. The initial diagnosis was carried out by real-time directed to the *m000.5L/R* gene, a method recommended by the OIE [23] to detect MYXV, and by a conventional PCR [12], followed by sequencing to confirm the presence of recombinant strains containing the disruptive insertion within the *m009L* gene.

4.7. EvaGreen-Based Multiplex Real-Time PCR (EG-mPCR)

The Taqman multiplex system was also optimized for the SsoFast™ EvaGreen® Supermix amplification kit (Bio-Rad, Lisbon, Portugal). The conditions were the following for a final 20 μL reaction: 10 μL of SsoFast EvaGreen supermix, 0.5 μL of each forward primer (0.08 to 0.12 μM, Table 3), 0.5 μL of reverse primer (0.08 to 0.12 μM, Table 3), 5 μL of DNA template and 4 μL of RNase/DNase free water.

Amplifications were run on a CFX96 real-time system associated with the C1000 thermal cycle (BIORAD), under the following conditions: an initial denaturation step at 98 °C for 2 min followed by 39 cycles of denaturation at 98 °C for 5 s, and annealing and extension at 60 °C for 15 s. Finally, the melting curve was analysed at a range of temperatures of 65–95 °C (with 0.3 °C increments), 5 s/step. The melting temperature of each amplicon (*m000.5L/R*, *m009L* and *m060L*) was initially determined by uniplex and then validated to the multiplex.

References

1. Myers, N.; Mittermeier, R.A.; Mittermeier, C.G.; Fonseca, G.A.B.; Kent, J. Biodiversity hotspots for conservation priorities. *Nature* **2000**, *403*, 853–858. [CrossRef] [PubMed]
2. Mittermeier, R.A.; Gil, P.R.; Hoffman, M.; Pilgrim, J.; Brooks, T.; Mittermeier, C.G.; Lamoreux, J.; da Fonseca, G.A.B. *Hotspots Revisited: Earth's Biologically Richest and Most Endangered Terrestrial Ecoregions*; Conservation International: Mexico City, Mexico, 2005.
3. WWF. *Living Planet Report 2020-Bending the Curve of Biodiversity Loss*; Almond, R.E.A., Grooten, M., Petersen, T., Eds.; WWF: Gland, Switzerland, 2020; ISBN 9782940529995.
4. Stuart Chapin, F.; Zavaleta, E.S.; Eviner, V.T.; Naylor, R.L.; Vitousek, P.M.; Reynolds, H.L.; Hooper, D.U.; Lavorel, S.; Sala, O.E.; Hobbie, S.E.; et al. Consequences of changing biodiversity. *Nature* **2000**, *405*, 234–242. [CrossRef] [PubMed]
5. Cowling, R.M.; Rundel, P.W.; Lamont, B.B.; Arroyo, M.K.; Arianoutsou, M. Plant diversity in mediterranean-climate regions. *Trends Ecol. Evol.* **1996**, *11*, 362–366. [CrossRef]
6. Médail, F.; Quézel, P. Biodiversity Hotspots in the Mediterranean Basin: Setting Global Conservation Priorities. *Conserv. Biol.* **1999**, *13*, 1510–1513. [CrossRef]
7. Delibes-Mateos, M.; Delibes, M.; Ferreras, P.; Villafuerte, R. Key role of European rabbits in the conservation of the western Mediterranean Basin hotspot. *Conserv. Biol.* **2008**, *22*, 1106–1117. [CrossRef]
8. Fenner, F. Portraits of Viruses: The Poxviruses. *Intervirology* **1979**, *11*, 137–157. [CrossRef]
9. Moss, B. Poxviridae: The viruses and their replication. In *Fundamental Biology*; Fields, B.N., Knipe, D.M., Howley, P.M., Eds.; Lippincott-Raven Publishers: Philadelphia, PA, USA, 1996.
10. Cameron, C.; Hota-Mitchell, S.; Chen, L.; Barrett, J.; Cao, J.X.; Macaulay, C.; Willer, D.; Evans, D.; McFadden, G. The complete dna sequence of myxoma virus. *Virology* **1999**, *264*, 298–318. [CrossRef] [PubMed]
11. Morales, M.; Ramírez, M.A.; Cano, M.J.; Párraga, M.; Castilla, J.; Pérez-Ordoyo, L.I.; Torres, J.M.; Bárcena, J. Genome Comparison of a Nonpathogenic Myxoma Virus Field Strain with Its Ancestor, the Virulent Lausanne Strain. *J. Virol.* **2009**, *83*, 2397–2403. [CrossRef]
12. Dalton, K.P.; Martín, J.M.; Nicieza, I.; Podadera, A.; Llano, D.; Casais, R.; Gimenez, S.; Badiola, I.; Agüero, M.; Duran, M.; et al. *Myxoma virus* jumps species to the Iberian hare. *Transbound. Emerg. Dis.* **2019**, *66*, 2218–2226. [CrossRef]
13. Pinto, A.; de Matos, A.L.; Abrantes, M.; Kraberger, S.; Risalde, M.A.; Gort, C.; Mcfadden, G.; Varsani, A.; Esteves, P.J. Genetic Characterization of a Recombinant Myxoma Virus in the Iberian Hare (*Lepus granatensis*). *Viruses* **2019**, *11*, 530. [CrossRef]
14. García-Bocanegra, I.; Camacho-Sillero, L.; Caballero-Gómez, J.; Aguero, M.; Gómez-Guillamón, F.; Ruiz-Casas, J.; Díaz-Cao, J.; García, E.; Ruano, M.; Haza, R. Monitoring of emerging myxoma virus epidemics in Iberian hares (*Lepus granatensis*) in Spain, 2018–2020. *Transbound. Emerg. Dis.* **2021**, *68*, 1275–1282. [CrossRef]
15. Carvalho, C.; Abade Dos Santos, F.A.; Monteiro, M.; Carvalho, P.; Mendonça, P.; Duarte, M.D. First cases of myxomatosis in Iberian hares (*Lepus granatensis*) in Portugal. *Vet. Rec. Case Rep.* **2020**, *8*, e001044. [CrossRef]
16. Duarte, M.D.; Carvalho, C.L.; Abade dos Santos, F.A.; Gomes, J.; Alves, P.C.; Esteves, P.J.; Abrantes, J.; Lopes, A.M.; Lopes, P.M.; Serronha, A.; et al. *+Coelho 2: Desenvolvimento e Implementação de Medidas Práticas Impulsionadoras da Recuperação dos Leporídeos Silvestres em Portugal*; INIAV: Oeiras, Portugal, 2021.
17. Carvalho, C.L.; Abade dos Santos, F.A.; Fagulha, T.; Carvalho, P.; Mendonça, P.; Monteiro, M.; Duarte, M.D. Myxoma virus and rabbit haemorrhagic disease virus 2 coinfection in a European wild rabbit (*Oryctolagus cuniculus algirus*), Portugal. *Vet Rec. Case Rep.* **2020**, *8*, e001002. [CrossRef]
18. Abade dos Santos, F.A.; Monteiro, M.; Pinto, A.; Carvalho, C.L.; Peleteiro, M.C.; Carvalho, P.; Mendonça, P.; Carvalho, T.; Duarte, M.D. First description of a herpesvirus infection in genus Lepus. *PLoS ONE* **2020**, *14*, 1–20. [CrossRef]
19. Duarte, M.D.; Carvalho, C.; Abade dos Santos, F.A.; Monteiro, J.; Monteiro, M.; Carvalho, P.; Mendonça, P.; Tavares Santos, P.; Melo, P. The Health and Future of the Six Hare. In *Lagomorphs*; IntechOpen: London, UK, 2020. (In press)
20. Abade dos Santos, F.; Carvalho, C.; Pinto, A.; Rai, R.; Monteiro, M.; Carvalho, P.; Mendonça, P.; Peleteiro, M.C.; Parra, F.; Duarte, M.D. Detection of Recombinant Hare Myxoma Virus in Wild Rabbits (*Oryctolagus cuniculus algirus*). *Viruses* **2020**, *12*, 1127. [CrossRef] [PubMed]
21. Abade dos Santos, F.A.; Carvalho, C.L.; Monteiro, M.; Carvalho, P.; Mendonça, P.; Peleteiro, M.C.; Duarte, M.D. Recombinant myxoma virus infection associated with high mortality in rabbit farming (*Oryctolagus cuniculus*). *Transbound. Emerg. Dis.* **2020**, *68*, 2616–2621. [CrossRef]
22. Soriguer, R.; Carro, F. Lepus granatensis. In *The IUCN Red List of Threatened Species 2019: E.T41306A2953195*; IUCN: Gland, Switzerland, 2019.
23. Duarte, M.D.; Barros, S.C.; Henriques, A.M.; Fagulha, M.T.; Ramos, F.; Luís, T.; Fevereiro, M. Development and validation of a real time PCR for the detection of myxoma virus based on the diploid gene M000. 5L/R. *J. Virol. Methods* **2014**, *196*, 219–224. [CrossRef]
24. Kerr, P.J.; Rogers, M.B.; Fitch, A.; DePasse, J.V.; Cattadori, I.M.; Hudson, P.J.; Tscharke, D.C.; Holmes, E.C.; Ghedin, E. Comparative Analysis of the Complete Genome Sequence of the California MSW Strain of Myxoma Virus Reveals Potential Host Adaptations. *J. Virol.* **2013**, *87*, 12080–12089. [CrossRef] [PubMed]
25. Kuchipudi, S.V.; Tellabati, M.; Nelli, R.K.; White, G.A.; Perez, B.B.; Sebastian, S.; Slomka, M.J.; Brookes, S.M.; Brown, I.H.; Dunham, S.P.; et al. 18S rRNA is a reliable normalisation gene for real time PCR based on influenza virus infected cells. *Virol. J.* **2012**, *9*, 230. [CrossRef]

26. Wang, Y.; Das, A.; Zheng, W.; Porter, E.; Xu, L.; Noll, L.; Liu, X.; Dodd, K.; Jia, W.; Bai, J. Development and evaluation of multiplex real-time RT-PCR assays for the detection and differentiation of foot-and-mouth disease virus and Seneca Valley virus 1. *Proc. Natl. Acad. Sci. USA* **2019**, *67*, 1–13. [CrossRef]

27. Wada, H. Details of the evolutionary history from invertebrates to vertebrates, as deduced from the sequences of 18S rDNA. *Proc. Natl. Acad. Sci. USA* **1994**, *91*, 1801–1804. [CrossRef]

28. Abade dos Santos, F.A.; Carvalho, C.L.; Peleteiro, M.C.; Parra, F.; Duarte, M.D. A Versatile qPCR for Diagnosis of Leporid Gammaherpesvirus 5 Using Evagreen® or Taqman® Technologies. *Viruses* **2021**, *13*, 715. [CrossRef] [PubMed]

29. So, K.Y.K.; Fong, J.J.; Lam, I.P.Y.; Dudgeon, D. Pitfalls during in silico prediction of primer specificity for eDNA surveillance. *Ecosphere* **2020**, *11*, e03193. [CrossRef]

30. van Weezep, E.; Kooi, E.A.; van Rijn, P.A. PCR diagnostics: In silico validation by an automated tool using freely available software programs. *J. Virol. Methods* **2019**, *270*, 106–112. [CrossRef]

31. OIE. Terrestrial Manual Myxomatosis. In *Lagomorpha*; OIE: Paris, France, 2018; pp. 1371–1388.

32. Bocanegra, I.G.; Camacho-Sillero, L.; Risalde, M.A.; Dalton, K.; Caballero-Gómez, J.; Aguero, M.; Zorrila, I.; Gómez-Guillamón, F. First outbreak of myxomatosis in Iberian hares (*Lepus granatensis*). *Transbound. Emerg. Dis.* **2019**, *66*, 2204–2208. [CrossRef]

33. Dalton, K.P.; Nicieza, I.; Baragão, A.; Alonso, J.M.M.; Parra, F. Molecular characterisation of virulence graded field isolates of myxoma virus. *Virol. J.* **2010**, *7*, 49. [CrossRef] [PubMed]

34. Duarte, M.D.; Carvalho, C.L.; Abade dos Santos, F.A.; Gomes, J.; Alves, P.C.; Esteves, P.J.; Abrantes, J.; Lopes, A.M.; Lopes, P.M.; Serronha, A.; et al. *+Coelho: Avaliação Ecossanitária das Populações Naturais de Coelho Bravo Visando o Controlo da Doença Hemorrágica Viral*; INIAV: Oeiras, Portugal, 2018.

How to detect and quantify the LeHV-5 in the context of laboratory diagnosis?

Scientific publication 5

A Versatile qPCR for Diagnosis of Leporid Gammaherpesvirus 5 Using Evagreen® or Taqman® Technologies

■ ■

Further to the description of leporid gammaherpesviruses (LeHV-5), there was a need to develop a specific, highly sensitive, quantitative method for monitoring and research. A real-time PCR was developed, to be used in TaqMan or Evagreen system, normalized by housekepping gene 18S rRNA.

MDPI

Article

A Versatile qPCR for Diagnosis of Leporid Gammaherpesvirus 5 Using Evagreen® or Taqman® Technologies

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Abstract: In late 2019, the first herpesvirus in the genus Lepus, named leporid gammaherpesvirus 5 (LeHV-5) was described. At the time, herpetic typical lesions were observed in hares infected by the myxoma virus, which is known to induce immunosuppression. Though the real impact of LeHV-5 is still poorly understood, since it affects reproduction, it poses an additional threat to the already fragile populations of Iberian hare, demanding prevalence investigations. In this article, we describe the first quantitative molecular method for LeHV-5 detection, using either Taqman or the EvaGreen systems. This method has excellent sensitivity and specificity, it is able to detect 2.1 copies of LeHV-5 DNA and was validated with an internal control targeting the 18S rRNA gene, allowing monitoring extraction and PCR amplification efficiencies.

Keywords: herpesvirus; gammaherpesvirus; leporid gammaherpesvirus 5; *Lepus granatensis*; Iberian hare; reproduction; qPCR

1. Introduction

The Iberian hare (*Lepus granatensis*) solely inhabits the Iberian Peninsula and is currently considered a "least concern" species by the International Union for Conservation of Nature, although a declining trend was recognized in the 2019 review [1,2]. The Iberian hare is the only hare species found in Portugal and in the last years, many local populations became extinct or significantly reduced due to cumulative changes in the habitat and emergence of new pathogens. Multidisciplinary approaches to investigate the causes of mortality and decline of the Iberian hare and European rabbit (*Oryctolagus cuniculus*), both key wild species of the Mediterranean ecosystems, is paramount for biodiversity conservation [1,3].

There are no studies evaluating the different causes of the decline of the Iberian hare populations, and the health status of this species is less well-known than that of rabbits. Recently, researchers became more interested in the Iberian hare after the emergence of myxomatosis [4,5] in 2018. Until then, myxomatosis only affected rabbits, and as reported, other species of hares [6–8].

Very recently, leporid gammaherpesvirus 5 (LeHV-5), the first herpesvirus found in hares, was described [9], but its real impact on the population is still difficult to evaluate or anticipate. However, the occurrence of genitalia necrosis, one of the lesions associated with LeHV-5 infection, shows its inevitable impact on reproductive activity and birth rate. LeHV-5 was also detected in apparently healthy hares, suggesting that LeHV-5 is circulating in the population, as observed with other human gammaherpesvirus (e.g., Epstein-Barr virus). Additionally, the high number of symptomatic co-infections by LeHV-5 and the

recombinant myxoma virus (ha-MYXV) [1,9] suggests that immunosuppression caused by MYXV triggers the necessary mechanisms for reactivation of LeHV-5 in asymptomatic infected hares. Given the fragile state of Iberian hare populations in Portugal and Spain after mid-2018, and since ha-MYXV is endemic in the Iberian Peninsula, it is important to investigate the LeHV-5 geographic spread in wild populations, and to assess the overlap with areas where MYXV epidemics are recurrent.

To improve the detection of LeHV-5, a real-time PCR system targeting the LeHV-5 glycoprotein B encoding gene was developed, validated, and optimized, to be used by TaqMan or EvaGreen methods.

To avoid false results, we normalized the qPCR method to detect failures in the extraction process and amplification reaction due to inhibitor factors. The internal reference chosen was the 18S rRNA housekeeping gene, widely described as a reliable normalization gene [10]. The method proved to be a highly sensitive and specific tool to diagnose LeHV-5 in a single analysis, through a fast and simple process.

Monitoring LeHV-5 and MYXV would allow us to understand their mutual inference and to elucidate the pathophysiology of the two infections.

2. Materials and Methods

2.1. Biological Samples

No animals were scarified for this study as only hares found dead in the field were used. Carcasses arrived at the Portugal National Reference Laboratory (INIAV, I.P.) within the scope of the +Coelho Project (Dispatch no. 4757/2017 of 31 May). During the necropsies, samples of liver and spleen were collected for virology investigations and preserved at −20 °C, until analysis.

2.2. DNA Extraction

For nucleic acid extraction, fresh samples of liver and spleen were homogenised at 20% (*w/v*) with phosphate buffered saline (PBS) and clarified at 3000 g for 5 min. Total DNA and RNA were extracted from 200 μL of the clarified supernatants, using the MagAttract 96 cador Pathogen Kit (Qiagen, Hilden, Germany) in a BioSprint 96 nucleic acid extractor (Qiagen, Hilden, Germany), according to the manufacturer's protocol. The nucleic acids were preserved at −20 °C until use.

2.3. Primer Design

The primers were designed based on LeHV-5 gB nucleotide sequences obtained previously (Abade dos Santos et al., 2020) (MN557129 to MN557133), using PCR Primer Design software (Eurofins Genomics, Ebersberg, Germany) but manually optimised. The nucleotide sequences of the primers and probe are described in Table 1. The target regions for the primers and probe were preserved in all samples analysed (*n* = 20, results not shown). The size of the amplicon generated was 200 bp.

Table 1. Primers and probes designed for the qPCR systems.

Gene Targeted	Primers (Name/Nucleotide Sequence (5′–3′))	Probe (Name/Fluorophore/Sequence (5′–3′)/Quencher)
gB LeHV-5	**LeHV-5-gB Fw** GGACTCAGTGAACATTCACCAAAGCC **LeHV-5-gB Rv** CACCCACGATAAAAAAGTGCTCTGCC	**LeHV-5-gB_P** *[Texas Red]* TGCTCTCCAACACGCAGCTCGAAACATGCC *[BHQ1]*
18S rRNA	**18S Fw** TATGGTTCCTTTGGTCGCTCGCTC **18S Rv** TCTGATAAATGCACGCATCCCCCC	**18S Prb** *[CY5]* AGCTAATACATGCCGACGGGCGCTGACC *[BHQ2]*

To be included as a dual internal extraction and amplification control in the duplex qPCR, we designed two primers within the 18S ribosomal RNA gene, using a partial 18S rRNA gene sequence of *Lepus* europaeus (AY150540, GenBank). Primers were validated in silico and in vitro.

2.4. Cloning

The amplicons generated by the LeHV-5gB and 18S gene conventional PCR systems (Table 1) were visualized by agarose gel electrophoresis, and the reaction product was purified and directly cloned into the pCR2.1 TA vector (Invitrogen Corporation, San Diego, CA, USA), according to the manufacturer's recommendations.

Transformation of the *Escherichia coli* competent cells (one-shot TOP 10, Invitrogen) was carried out as recommended. White colonies were isolated and inoculated in 5 mL of Luria Broth medium and grown overnight at 37 °C with 220 rotations per minute. Recombinant DNA was obtained using the NZYMiniprep Kit (NZYTech, Lisbon, Portugal). Three recombinant plasmids containing partial sequences from the LeHV-5 gB, 18S hare, and 18S rabbit were purified from the bacterial cultures.

Both DNA strands of the pgB, p18S_ha (*Lepus granatensis*) and p18S_r (*Oyctolagus cuniculus algirus*) inserts were sequenced using the M13F and M13R primers and a 3130 Genetic Analyser (Applied Biosystems, Foster City, CA, USA). The 117bp sequences from p18S_ha and p18S_r were aligned and used to design the probe (Table 1). Sequences were assembled and edited using the Bioedit 7.2 software (version?).

2.5. Evaluation of the PCR Component Variation

The LeHV-5 and 18S rDNA qPCR systems were first optimized separately by analysing the impact of variation of several parameters in the PCR performance. The main variables for qPCR amplification were crossed, to identify each optimal condition, namely the annealing temperature (52 to 62 °C), the dNTP concentration (0.2 mM to 0.4 mM), the $MgCl_2$ final concentration (2.5 mM to 6 mM), and the primer concentration (0.1 and 0.3 μM). These combinations were tested using 10^8 and 10 copies of pgB or p18S_ha. The NZYTaq II 2× Colourless Master Mix (NZYTech, Lisbon, Portugal) was used in these assays, adapting the dNTP and $MgCl_2$ concentrations with standard solutions.

The final reaction volume was 25 μL, including 0.5 μL of each forward primer (variable final concentrations), 0.5 μL of each reverse primer (variable final concentrations), 0.5 μL of each probe (fixed 0.2 μM final concentration) and 5 μL of the DNA template. RNase/DNase free water was used to make up the final volume. The amplification was run on a CFX96 real-time system associated with C1000 thermal cycle (BIORAD) under the following conditions—initial denaturation at 95 °C for 3 min followed by 39 cycles of denaturation at 94 °C for 30 s; variable annealing temperature for 30 s and extension at 72 °C for 30 s. These combinations were also used for testing the interference between the two pairs of primers and the two probes, by analysing the effects in the Ct and Fluorescence values.

2.6. Field Samples Analysis

For field samples, the final reaction volume was 25 μL for the Taqman system, which comprised 12.50 μL of Multiplex PCR NZYTaq 2× Colourless Master Mix, 0.5 μL of each forward primer (0.2 μM final concentration), 0.5 μL of each reverse primer (0.2 μM final concentration), 0.5 μL of each probe (0.2 μM final concentration), 5 μL of DNA template from each sample, and 4.5 μL RNase/DNase free water. The amplification was run on a CFX96 real-time system associated with the C1000 thermal cycle (BIORAD) under the following conditions—initial denaturation at 95 °C for 3 min followed by 49 cycles of denaturation at 94 °C for 30 s, annealing at 62 °C for 30 s and extension at 72 °C for 30 s.

For the EvaGreen system, the final reaction volume was 20 μL comprising 10 μL of SsoFast™ EvaGreen® Supermix, 0.5 μL of forward primer (0.2 μM final concentration), 0.5 μL of reverse primer (0.2 μM final concentration), 5 μL of DNA template from each sample, and 6.5 μL RNase/DNase free water. The amplification was run on a CFX96

real-time system associated with the C1000 thermal cycle (BIORAD), under the following conditions—initial denaturation at 98 °C for 2 min followed by 39 cycles of denaturation at 98 °C for 5 s, annealing and extension at 62 °C for 5 s. Finally, the melt curve was analyzed at a range of temperatures of 65–95 °C (0.5 °C increment), 5 s/step. In this case, the 18S internal control was not incorporated, in order to avoid confusing the result of the amplification curve. However, a High Resolution Melt Analysis allowed for its inclusion (results not shown).

2.7. Detection Limit, Sensitivity, Specificity, Repeatability, and Reproducibility of the qPCR

The specificity of the primers and probes of the LeHV-5 gB and 18S rDNA qPCR systems were first determined in silico using the BLASTN analysis service from NCBI. To further extend the specificity evaluation of the method, total genomes (or the glycoprotein B gene in case of some herpesviruses) of potential rabbit and hare pathogens were tested against the LeHV-5 primers and probe, with very relaxed temperature conditions (namely showing all matching sites of primer binding, a length of 60 bp to 3000 bp and allowing mismatches in 5 nucleotides of the 3′end), looking for all possible connection points using the software FASTPCR 6.7 (PrimerDigital, 2020).

The genomes tested included *Bibersteinia trehalosi* (NZ_CP006954), *Chlamydophila abortus* (CR848038), *Coxiella burnetii* (CP040059), *Cryptosporidium parvum* (CM000436), *Escherichia coli* (AE014075), *Encephalitozoon cuniculi* (LFTZ0100z0003), *Enterococcus faecalis* (CP045918), *Francisella tularensis* (CP025778), *Klebsiella pneumoniae* (FO203501), *Leptospira interrogans* (CP039256), *Mannheimia haemolytica* (CP006957), *Pasteurella multocida* (CP031552), *Salmonella enterica* (CP003278), *Serratia* sp. (CP025085), *Staphylococcus aureus* (AP017922), *Staphylococcus epidermidis* (CP043847), *Toxoplasma gondii* (U87145), *Myxoma virus* (KP723389 and KP723390), and *ha-MYXV* (MK340973), *and other species of herpesvirus that do not affect hares, including Macacine herpesvirus 5 (NC_003401), Human herpesvirus 2 (KU310663), Macaca mulatta rhadinovirus (AF210726), Gorilla rhadinovirus 1 (AY177144), Rodent herpesvirus (NC_015049), Apodemus agrarius rhadinovirus (AY854168), Mus cervicolor rhadinovirus 1 (DQ821582), Apodemus flavicollis rhadinovirus 1 (DQ821580), and Microtus agrestis rhadinovirus 1 (EF128052).* Some RNA viruses that might not be amplified by this DNA-based qPCR system, were also evaluated given that they might be included in a multiplex RNA/DNA PCR System in the future. These included hare lagovirus (KR230102, MK138384), RHDV (MF421574), and the European brown hare syndrome virus (MK440616).

To assess the specificity of the method, the system was also tested in vitro (both as a uniplex and duplex) using samples of the total nucleic acid extracted from animal tissues infected with the following species of viruses, *bovine herpesvirus* (MCFV, BoHV-1 and BoHV-4), *Equine herpesvirus* (EHV-1, EHV-2 and EHV-5), *Canine herpesvirus* (CaHV-1), *Felid herpesvirus* (FeHV-1), *Gallid herpesvirus 1* (GaHV-1) and 2 (GaHV-2), *Psittacid herpesvirus 1* (PDV), myxoma virus (from rabbit and Iberian hare), rabbit haemorrhagic disease virus, total nucleic acid of rabbit, and hare isolated colonies of *E.coli*, *P. multocida*, and *S. aureus*, and total nucleic acid from the isolated parasites of *Cysticercus pisiformis*, *Eimeria stiedae*, and *Eimeria* spp. and *Passalurus ambiguous,* available at the National Reference Laboratory for Animal Health (INIAV, I.P, Oeiras, Portugal). In all reactions, 20 ng of the template was used. The PCRs were performed using the conditions described above in this manuscript, along with a more relaxed annealing temperature of 55 °C.

DNA concentrations of plasmid pgB, p18S_ha, and p18S_r were determined by the A260 measurement (Qubit 4 Fluorimeter by Invitrogen, California, USA), and the copy number was calculated based on the plasmid molecular weight (3929 bp). A series of tenfold dilution of plasmid incorporating the insert were prepared in sterile ddH$_2$O. These serial dilutions were primarily used to determine the limit of detection (LOD) for each system.

Standard curves were constructed with the PCR results from three replicates per dilution, by plotting the crossing point values (Ct value) against the logarithm of the DNA

copy number. Average values and standard deviation for the crossing point values were also calculated. The absolute copy number of the plasmid in relation to the starting amount of DNA was calculated accordingly to the formula: number of copies detected = amount of DNA(g) $\times$ 6.022 $\times$ 10^{23}/fragment(bp) $\times$ 650.

2.8. Validation of 18S DNA as Internal Control

To evaluate the robustness of the Iberian hare 18S rRNA housekeeping gene regarding its resistance to degradation, we extracted samples from brain, skin, heart, lung, duodenum, kidney, liver, and genital samples, and preserved them in different conditions. qPCR tests were carried out immediately after extraction, two weeks after freezing at $-20\ ^\circ$C (without nucleic acids protection solutions), and two weeks after incubation at room temperature (without nucleic acids protection solutions) to assess the resistance of the internal control DNA. All tests were performed in duplicates.

2.9. Clinical Samples Validation

To assess the sensitivity and specificity of the method for LeHV-5 DNA detection in field samples, several hare samples available in the National Reference Laboratory of Portugal (INIAV, I.P.) were used. These samples were first analyzed with a nested PCR system [11] using the NZYTaq II 2$\times$ Colourless Master Mix (NZYTech, Lisbon, Portugal) and subsequently tested with the duplex PCR for LeHV-5 gB and 18S rDNA, using the Multiplex PCR NZYTaq 2$\times$ Colourless Master Mix (NZYTech, Lisbon, Portugal).

3. Results

3.1. Specificity of the Method

The tests performed in silico and in vitro did not show amplification for any of the etiological agents tested (14 bacteria in silico and 3 bacteria in vitro, 3 parasites in silico and 4 parasites in vitro, and 16 viruses in silico and 13 viruses in vitro), demonstrated the specificity and robustness of the method (results not shown).

3.2. Efficiency and Sensitivity of the LeHV-5 Taqman System

A typical standard curve amplification plot and linear regression analysis for the LeHV-5 is shown in Figure 1. Excellent linearity was observed over nine orders of magnitude, from 2.2 $\times$ 10^8 copies to 2.1 $\times$ 10^0 copies (Figure 1A). The regression analysis for this interval yielded an R2 (correlation coefficient) of 0.998 and a y-intercept value of 37.26. The slope of 3.323 revealed a high qPCR efficiency (100.0%) (Figure 1B). The minimum number of copies of LeHV-5 DNA detected by the qPCR LeHV-5 gB system were 2.1 per 25 µL of reaction.

(A)   **(B)**
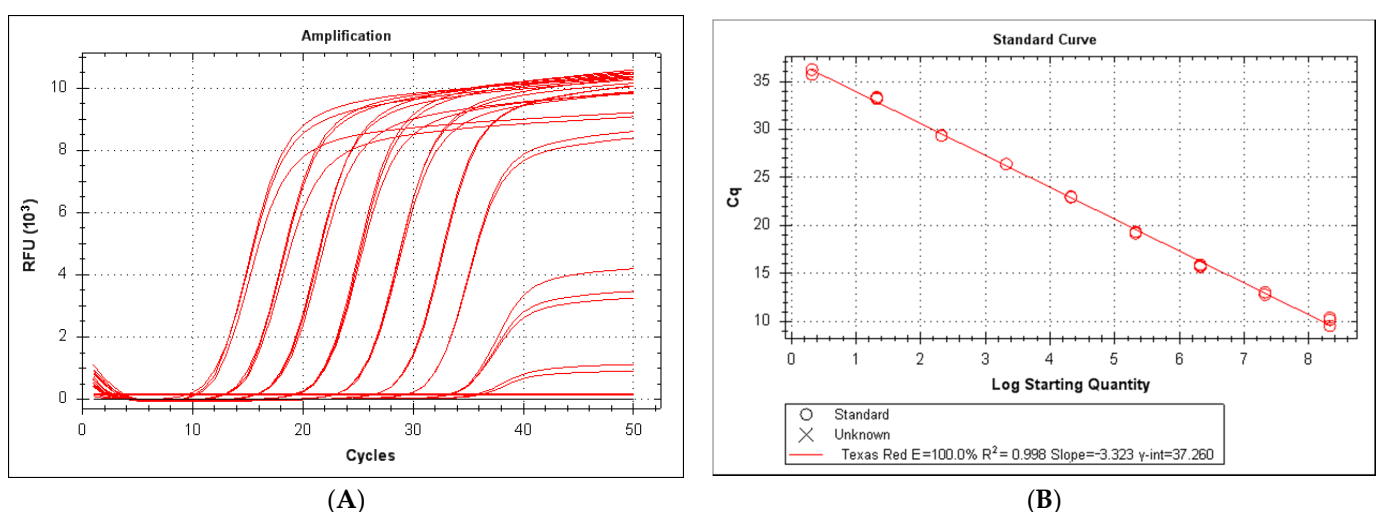
Figure 1. (**A**) Serial dilution from 2.1 $\times$ 10^8 to 2.1 copies per reaction. Black lines correspond to negative Scheme 2. 1 $\times$ 10^{-1} and 2.1 $\times$ 10^{-2} copies per reaction). (**B**) Standard curve from 2.1 $\times$ 10^8 to 2.1 copies per reaction.

3.3. Efficiency and Sensitivity of the LeHV-5 in the EvaGreen System

A standard curve was generated by plotting the threshold cycles of reference standards versus their log copy number. Linear regression of the reference concentrations yielded a correlation coefficient of 0.999 ($p < 0.001$). The minimum number of copies detected by the system was 2.1 copies per 20 μL reaction for LeHV-5, with an amplification efficiency of 99.7% (Figure 2A,B).

(A)

(B)

(C)

(D)
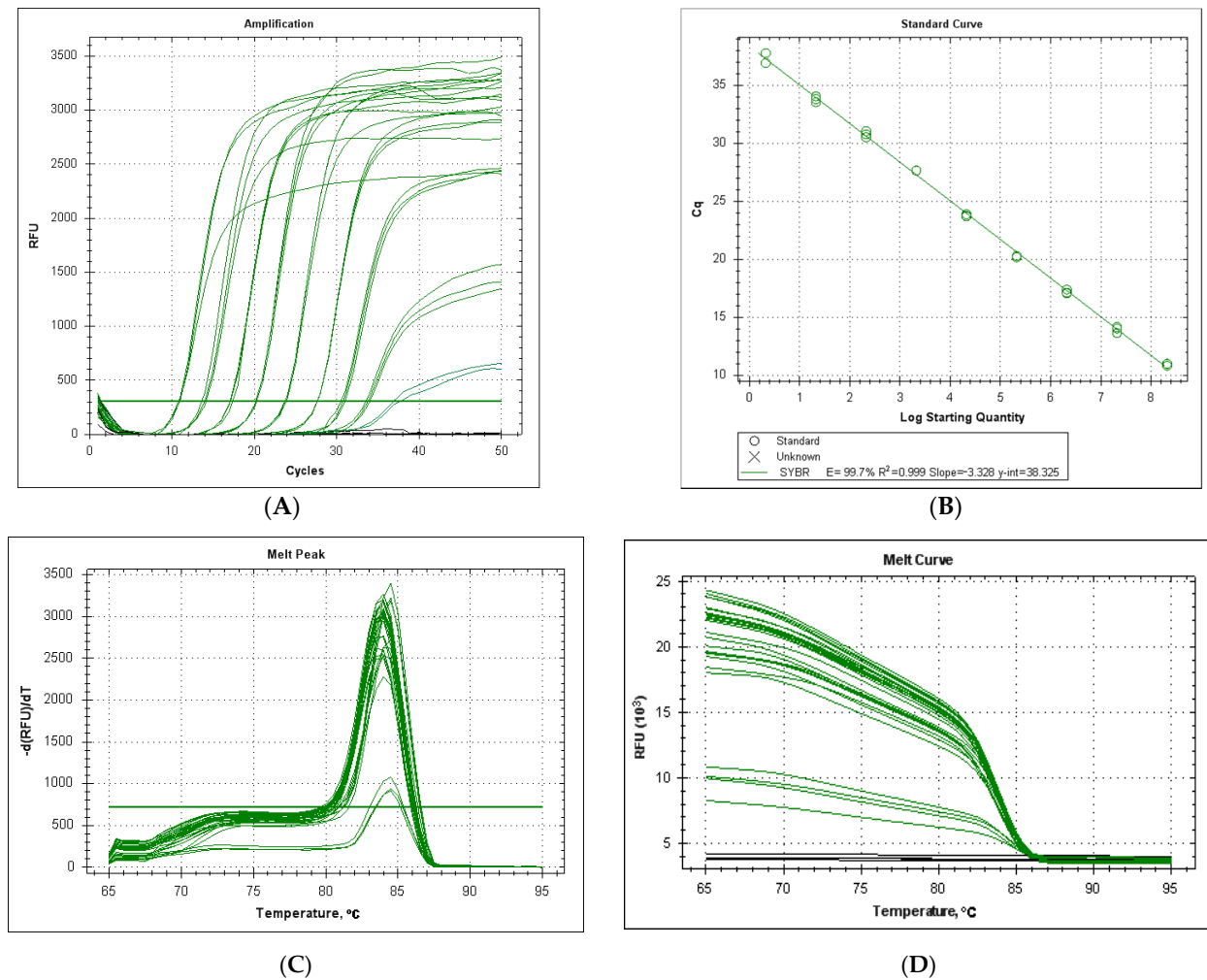
Figure 2. (**A**) Serial dilution from 2.1×10^8 to 2.1×10^0 copies per reaction. Black lines correspond to negative samples (2.1×10^{-1} and 2.1×10^{-2} copies per reaction). (**B**) Standard curve from 2.1×10^8 to 2.1×10^0 copies per reaction. (**C**) Melt point analysis for 36 reactions with 2.1×10^8 to 2.1×10^0 copies. (**D**) Melt curve analysis for 36 reactions with 2.1×10^8 to 2.1×10^0 copies.

As shown in Figure 2C, the melt curve analysis for the LeHV-5 gB amplifications showed a distinct unique peak in all reactions. The medium value for 36 analyses was 84.11 °C ± 0.21 °C. The variation in this temperature was more evident for the reactions with a lower number of DNA copies.

3.4. Behavior of 18S rDNA as an Internal Control

In silico analysis of the 18S rRNA gene showed that the designed system efficiently amplified a fragment of 117nt from Iberian hare and wild rabbit samples, as well as from several other mammalian species namely cat (*Felis catus),* harbor seal *(Phoca vitulina)*, cheetah *(Acinonyx jubatus)*, grizzly bear *(Ursus arctos horribilis)*, Steller sea lion *(Eumetopias jubatus)*, dingo *(Canis lupus dingo)*, dog *(Canis familiaris)*, ref fox *(Vulpes vulpes)* among others, with which a minimum identity of 99.15% was observed, according to the nucleotide sequences currently available in public databases.

The qPCR system for 18S amplification was robust when using different primer concentrations (Figure 3A). The minimum number of copies detected by the system was 12 copies per 25 μL reaction, with an amplification efficiency of 100% (Figure 3B,C).

(**A**)

(**B**)

(**C**)

(**D**)
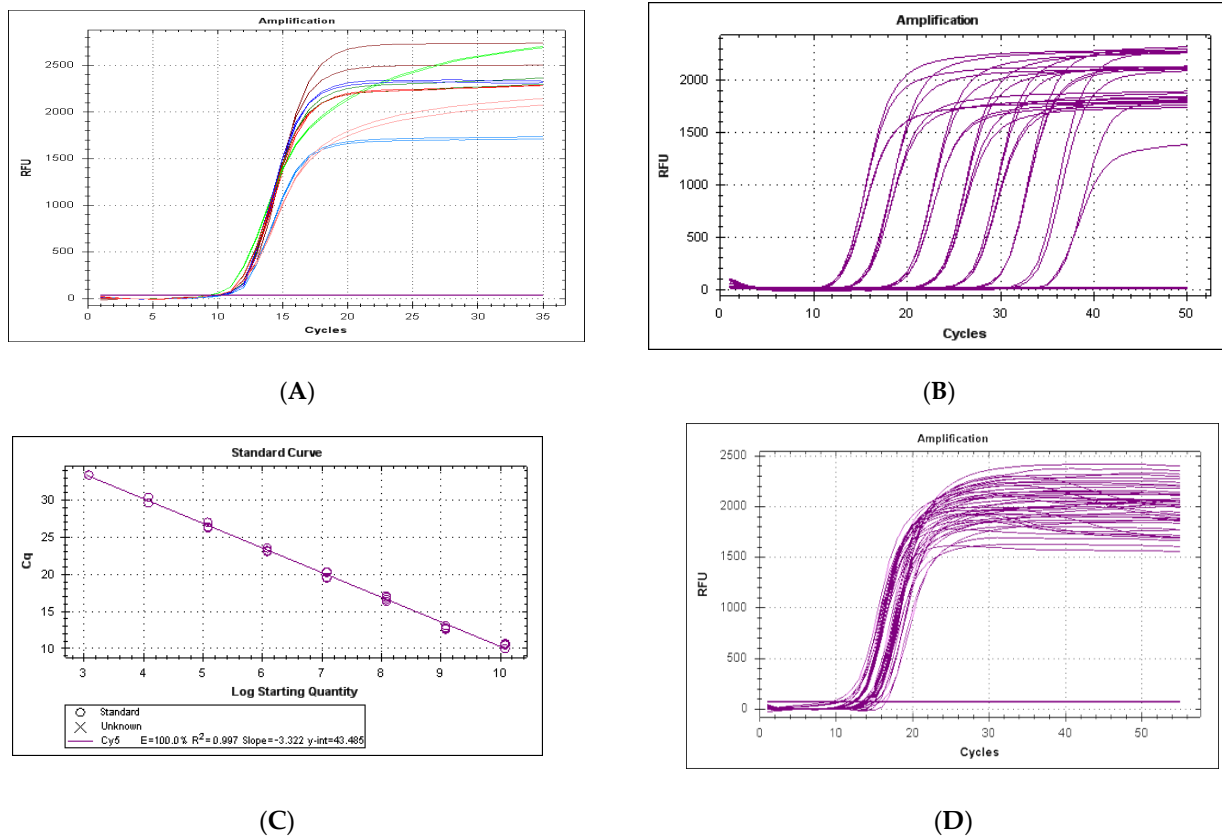
Figure 3. (**A**) Analysis of different primer concentrations for 18S DNA amplification. Fixed final concentration of the probe in all reactions (0.2 μM). Variable primer concentrations: 1 μM Fw primer, 1 μM Rev primer (brown), 0.5 μM Fw primer, 1 μM Rv primer (dark green), 0.2 μM Fw primer, 1 μM Rev primer (light green), 1 μM Fw primer, 0.5 μM Rev primer (dark blue), 0.5 μM Fw primer, 0.5 μM Rv primer (red), 0.2 μM Fw primer, and 0.2 μM Rv primer (orange). (**B**) Serial dilution from 1.2×10^8 to 1.2×10^1 copies of p18S_ha per reaction. Black lines correspond to lack of amplification (1.2×10^0 and 1.2×10^{-1} copies per reaction). (**C**) Standard curve from 1.2×10^8 to 1.2×10^1 copies per reaction. (**D**) Amplification of 40 samples of DNA (liver and spleen) from Iberian hare field samples.

The number of DNA copies detected in different field samples, homogenized at 20% (*w/v*) in PBS, was relatively consistent, ranging from Cq 9.16 to 16.24 (Figure 3D). When testing several samples without uniform homogenization (corresponding to 10–30% (*w/v*) final dilution in PBS), the 18S amplification Cq value ranged from 8.54 to 22.58.

To determine the robustness of the 18S qPCR, DNA was extracted from fresh hare samples and from the same samples after 7 days of incubation at 20–25 °C (RT) and at 37 °C. The Cq results are shown in Table 2, demonstrating that 18S rDNA was stable, even after one-week at 37 °C, showing a higher degradation in the liver, that could be justified by the parenchymal nature of this organ. The addition of antibiotic/antimycotic (AB/AM) had no obvious interference in the efficiency of DNA amplification, but the degree of liver putrefaction after 7 days was visible when compared to the Cq values obtained with the DNA extracted from a fresh liver sample. However, the putrefaction conditions tested had no relevant effect in the Cq values obtained in the lung and heart samples (Table 2).

3.5. Intra and Inter-Assay Reproducibility in the LeHV-5 Taqman and EvaGreen Systems

The reproducibility of the qPCR was evaluated with quadruplicates for each dilution replicated in two different assays. The Cq values for the intra-assay and inter-assay

variability evaluations are shown in Table 3. A maximum coefficient of variation (CV) of 2.47% and 2.43% was obtained. These values, both under 3.5%, revealed a high repeatability and reproducibility. All Taqman assays were performed with a stable quantity of 1.2×10^6 copies of p18S. In the case of EvaGreen, a singlexplex run was only carried out due to real-time differentiation limitations. However, a duplex using the EvaGreen system could be optimized and analyzed through high-resolution melt analysis.

Table 2. Cq values obtained with the 18S rDNA qPCR using European rabbit tissues submitted to different conservation/preservation conditions.

	Fresh			RT, 7 Days			37 °C, 7 Days		
Diluent	**Liver**	**Lung**	**Heart**	**Liver**	**Lung**	**Heart**	**Liver**	**Lung**	**Heart**
PBS	12.29	17.70	15.40	25.05	15.52	16.19	23.29	15.18	16.06
PBS, AB, AM	12.35	17.73	17.78	25.27	15.49	16.06	23.31	15.33	16.10

PBS (phosphate-buffered saline), AB—antibiotic, AM—antimycotic.

Table 3. Summary of statistical analyses of the repeatability and reproducibility results for both qPCR systems for gB detection.

	Taqman System					
Copies/Reaction	**Repeatability (Quadruplicates)**			**Repeatability (Three Independent Assays)**		
	Mean Cq	SD	%CV	Mean Cq	SD	%CV
2.1×10^8	10.73	0.181	1.69	10.32	0.185	1.79
2.1×10^7	13.44	0.093	0.69	13.55	0.101	0.75
2.1×10^6	16.31	0.061	0.37	16.10	0.070	0.43
2.1×10^5	20.03	0.086	0.43	19.86	0.084	0.42
2.1×10^4	23.47	0.114	0.49	22.85	0.104	0.46
2.1×10^3	27.31	0.080	0.29	27.44	0.082	0.30
2.1×10^2	30.45	0.222	0.73	30.11	0.256	0.85
2.1×10^1	34.07	0.159	0.47	34.05	0.175	0.51
2.1×10^0	36.36	0.897	2.47	37.13	0.904	2.43
	EvaGreen SYSTEM					
Copies/reaction	**Repeatability (Quadruplicates)**			**Repeatability (Three Independent Assays)**		
	Mean Cq	SD	%CV	Mean Cq	SD	%CV
2.1×10^8	10.19	0.076	0.75	10.67	0.090	0.84
2.1×10^7	13.31	0.074	0.56	13.80	0.114	0.83
2.1×10^6	17.18	0.073	0.42	16.89	0.094	0.56
2.1×10^5	20.70	0.188	0.91	20.14	0.250	1.24
2.1×10^4	24.29	0.269	1.11	23.98	0.314	1.31
2.1×10^3	27.91	0.044	0.16	27.50	0.098	0.36
2.1×10^2	31.30	0.248	0.79	31.05	0.322	1.04
2.1×10^1	34.89	0.177	0.51	34.60	0.198	0.57
2.1×10^0	37.63	0.491	1.30	37.40	0.503	1.34

CV—coefficient of variation.

3.6. Performance in the Diagnosis of Clinical Samples

The qPCR Taqman and EvaGreen systems allowed detection of a panel of positive samples, pre-diagnosed as LeHV-5 positive by the system described in [11], indicating a sensitivity of 100%. Likewise, both systems did not amplify any sample that was pre-classified as negative, showing a specificity of 100% (Table 4). All real-time PCR runs were validated by successful amplification of the 18S rRNA gene and sequencing.

Table 4. Comparative final diagnosis between Nested PCR [11] and Taqman rt-qPCR system.

Sample Code	Nested PCR *		Real Time (Cq)	Estimated Copies/mg Tissue *
	Amplification	Sequencing Analysis		
39189PT17	N	-	NA	NA
39190PT17	P	LeHV-5	20.23	7.43×10^6
39191PT17	N	-	NA	NA
39192PT17	P	LeHV-5	22.94	1.14×10^6
39344PT17	P	LeHV-5	24.24	4.61×10^5
39351PT17	N	-	NA	NA
39355PT17	N	-	NA	NA
00751PT18	N	-	NA	NA
00807PT18	N	-	NA	NA
00813PT18	P	LeHV-5	24.93	2.86×10^5
00814PT18	P	LeHV-5	23.87	5.96×10^5
00815PT18	P	LeHV-5	22.32	1.75×10^6
00816PT18	P	LeHV-5	25.62	1.77×10^5
00817PT18	P	LeHV-5	23.62	7.09×10^5
00818PT18	N	-	NA	NA
04971PT18	N	-	NA	NA
0492PT18	N	-	NA	NA
04483PT18	N	-	NA	NA
04985PT18	N	-	NA	NA
26935PT18	N	-	NA	NA
30901PT18	N	-	NA	NA
33014PT18	D	unspecific	NA	NA
33021PT18	D	unspecific	NA	NA
35866PT18	D	unspecific	NA	NA
35867PT18	D	unspecific	NA	NA
38457PT18	N	-	NA	NA
35869PT18	P	LeHV-5	21.06	4.18×10^6
38455PT18	P	LeHV-5	21.44	3.21×10^6
39375PT18	D	LeHV-5	26.10	1.27×10^5
41434PT18	D	unspecific	NA	NA
41439PT18	D	unspecific	NA	NA
41440PT18	D	unspecific	NA	NA
00129FPT19	D	unspecific	NA	NA
03230PT19	D	unspecific	NA	NA
4033PT19	P	LeHV-5	25.08	2.58×10^5
22059PT19	P	LeHV-5	26.45	9.97×10^4
4037PT19	P	LeHV-5	25.02	2.69×10^5
4064PT18	P	LeHV-5	14.27	4.63×10^8
24980PT19	D	unspecific	NA	NA
29959PT18	P	LeHV-5	25.19	2.39×10^5
1129PT18	P	LeHV-5	23.65	6.94×10^5
29973PT18	P	LeHV-5	25.71	1.67×10^5
30908PT18	D	herpesvirus	28.36	2.65×10^4
33023PT18	P	LeHV-5	22.57	1.47×10^6
30903PT18	P	LeHV-5	26.59	9.05×10^4

P—positive, N—negative, D—doubtful. NA (no amplification). * Estimative using the standard curve of Taqman system.

Ten samples were negative in the qPCR, while a weak band of compatible size was generated in the nested PCR (Table 4). This band was sequenced and confirmed to be unspecific.

Contrarily, two doubtful bands in the nested PCR, which were positive in the qPCR were sequenced and confirmed to be gammaherpesvirus.

The performance of the qPCR as a duplex (LeHV-5 and 18S), evaluated by the Ct value and the fluorescence intensity, did not interfere with the efficiency of the LeHV-5 detection compared to the singleplex, either using both plasmids (Figure 4A, both efficiencies of 100%) or field samples (Figure 4B). Cross interaction between the two probes and channels was tested and excluded by analyzing the results obtained in the method described in Section 2.4. The value of Cq and relative fluorescence showed no significant interference when comparing singleplex and duplex assays, while using different probe concentrations. For this reason, we decided to use the minimum concentration of primers and probes recommended by the commercial kit, making the method cheaper.

| **(A)** | **(B)** |
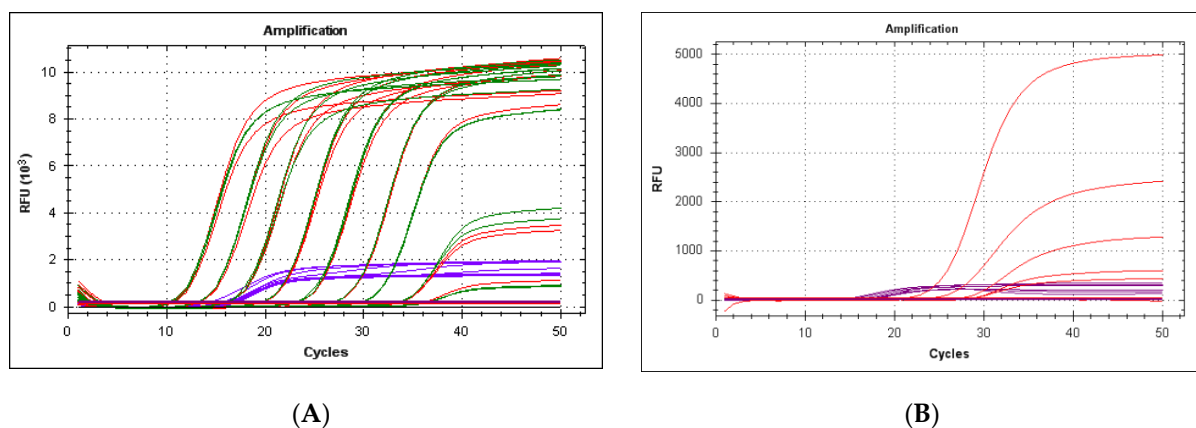
Figure 4. (**A**) Amplification of LeHV-5 gB gene in singleplex and duplex also for 18S rRNA gene (CY5 channel, purple curves). Red lines correspond to LeHV-5 amplification in singleplex reactions and green in duplex reactions. Horizontal black lines correspond to samples lacking amplification (2.1×10^{-1} and 2.1×10^{-2} copies per reaction) (**B**) Amplification of LeHV-5 (red curves) in duplex with the 18S rRNA gene (CY5 channel, purple curves), using field samples.

4. Discussion

The Iberian hare is an iconic species of the Iberian Peninsula, which has a tremendous ecological, cultural, and economic importance. The direct or indirect impact of LeHV-5, a herpesvirus recently described in this species, on the present and future stability of the hare populations [9] is unknown, aggravated by the lack of census data.

Given the high prevalence of this virus in the samples analysed so far (46.7%, results not shown) and the shortness of knowledge on the disease it causes, the understanding of the true impact and dispersion of the LeHV-5 infection relies on a specific diagnostic method for monitoring and surveillance. The LeHV-5/18S rRNA gene duplex system developed and validated (described here), allows for the quantification of this virus in different tissue samples, and provides a diagnosis tool to perceive its tropism and physiopathology.

The sensitivity and specificity of this method were of 100% when using samples previously classified as positive or negative from our biobank, tested by a nested PCR for mammalian and avian herpesviruses [11]. Two samples classified as negative/doubtful by this nested PCR system, were successfully detected by the LeHV-5 qPCR. Ten samples were not detected by the LeHV-5/18S rRNA gene system, despite a compatible band being generated on the nested PCR, but sequencing was found to be interspecific. The gB gene was previously used as a target for several real-time PCR systems for other herpesviruses [12,13]. Using the standard curve generated by this method, researchers would be able to quantify the viral copies presented in the tissues analysed and verify the extraction and amplification success by the 18S rDNA behaviour.

To our knowledge, this represents the first method for the molecular diagnosis of LeHV-5, a virus first reported just some months ago [9].

5. Conclusions

In this article, we present the first rapid and quantitative diagnosis method for leporid gammaherpesvirus 5 (LeHV-5). This method proved to be robust, highly sensitive and highly specific, and could be used in two different formats, according to the laboratory preferences, namely by hydrolysis of a Taqman probe or green fluorescent nucleic acid dying (*EvaGreen*). Considering that the virus was reported for the first time in 2018, retrospective and prospective studies are important to elucidate the extension of the virus' geographical spread in the hare populations. Finally, this study detected LeHV-5 in samples from 2017, providing evidence of the virus circulation before its first report in 2018.

References

1. Duarte, M.D.; Carvalho, C.; Abade dos Santos, F.A.; Monteiro, J.; Monteiro, M.; Carvalho, P.; Mendonça, P.; Tavares Santos, P.; Melo, P. The Health and Future of the Six Hare Species in Europe: A Closer Look at the Iberian Hare. In *Lagomorphs*; IntechOpen: London, UK, 2020; in press.
2. Soriguer, R.; Carro, F. Lepus granatensis. The IUCN Red List of Threatened Species 2019. Available online: https://dx.doi.org/10.2305/IUCN.UK.2019-1.RLTS.T41306A2953195.en (accessed on 13 April 2021).
3. Duarte, M.D.; Carvalho, C.L.; Abade dos Santos, F.A.; Gomes, J.; Alves, P.C.; Esteves, P.J.; Abrantes, J.; Lopes, A.M.; Lopes, P.M.; Serronha, A.; et al. +Coelho: Avaliação Ecossanitária das Populações Naturais de Coelho Bravo Visando o Controlo da Doença Hemorrágica Viral. Master's Thesis, University of Lisbon, Lisboa, Portugal, 2018.
4. Carvalho, C.; Abade Dos Santos, F.A.; Monteiro, M.; Carvalho, P.; Mendonça, P.; Duarte, M.D. First cases of myxomatosis in Iberian hares (Lepus granatensis) in Portugal. *Vet. Rec. Case Rep.* **2020**, *8*. [CrossRef]
5. Bocanegra, I.G.; Camacho-Sillero, L.; Risalde, M.A.; Dalton, K.; Caballero-Gómez, J.; Aguero, M.; Zorrila, I.; Gómez-Guillamón, F. First outbreak of myxomatosis in Iberian hares (Lepus granatensis). *Transbound. Emerg. Dis.* **2019**, 1–5. [CrossRef]
6. Wibbelt, G.; Frolich, K. Infectious Diseases in European Brown Hare (lepus europaeus). *Wildl. Biol. Pract.* **2005**, *1*. [CrossRef]

7.   Barlow, A.; Lawrence, K.; Everest, D.; Dastjerdi, A.; Finnegan, C.; Steinbach, F. Confirmation of myxomatosis in a European brown hare in Great Britain. *Vet. Rec.* **2014**, *175*, 75–76. [CrossRef] [PubMed]

8.   Collins, J.J. Myxomatosis in the common hare. *Ir. Vet. J.* **1955**, *9*, 268.

9.   Abade dos Santos, F.A.; Monteiro, M.; Pinto, A.; Carvalho, C.L.; Peleteiro, M.C.; Carvalho, P.; Mendonça, P.; Carvalho, T.; Duarte, M.D. First description of a herpesvirus infection in genus Lepus. *PLoS ONE* **2020**, *14*, 1–20. [CrossRef]

10.   Kuchipudi, S.V.; Tellabati, M.; Nelli, R.K.; White, G.A.; Perez, B.B.; Sebastian, S.; Slomka, M.J.; Brookes, S.M.; Brown, I.H.; Dunham, S.P.; et al. 18S rRNA is a reliable normalisation gene for real time PCR based on influenza virus infected cells. *Virol. J.* **2012**, *9*, 1. [CrossRef] [PubMed]

11.   Devanter, D.R.V.A.N.; Warrener, P.; Bennett, L.; Schultz, E.R.; Coulter, S.; Garber, R.L.; Rose, T.M.; Black, J. Detection and Analysis of Diverse Herpesviral Species by Consensus Primer PCR. *J. Clin. Microbiol.* **1996**, *34*, 1666–1671. [CrossRef] [PubMed]

12.   Decaro, N.; Amorisco, F.; Desario, C.; Lorusso, E.; Camero, M.; Bellacicco, A.L.; Sciarretta, R.; Lucente, M.S.; Martella, V.; Buonavoglia, C. Development and validation of a real-time PCR assay for specific and sensitive detection of canid herpesvirus 1. *J. Virol. Methods* **2010**, *169*, 176–180. [CrossRef] [PubMed]

13.   Wernike, K.; Hoffmann, B.; Kalthoff, D.; König, P.; Beer, M. Development and validation of a triplex real-time PCR assay for the rapid detection and differentiation of wild-type and glycoprotein E-deleted vaccine strains of Bovine herpesvirus type 1. *J. Virol. Methods* **2011**, *174*, 77–84. [CrossRef] [PubMed]
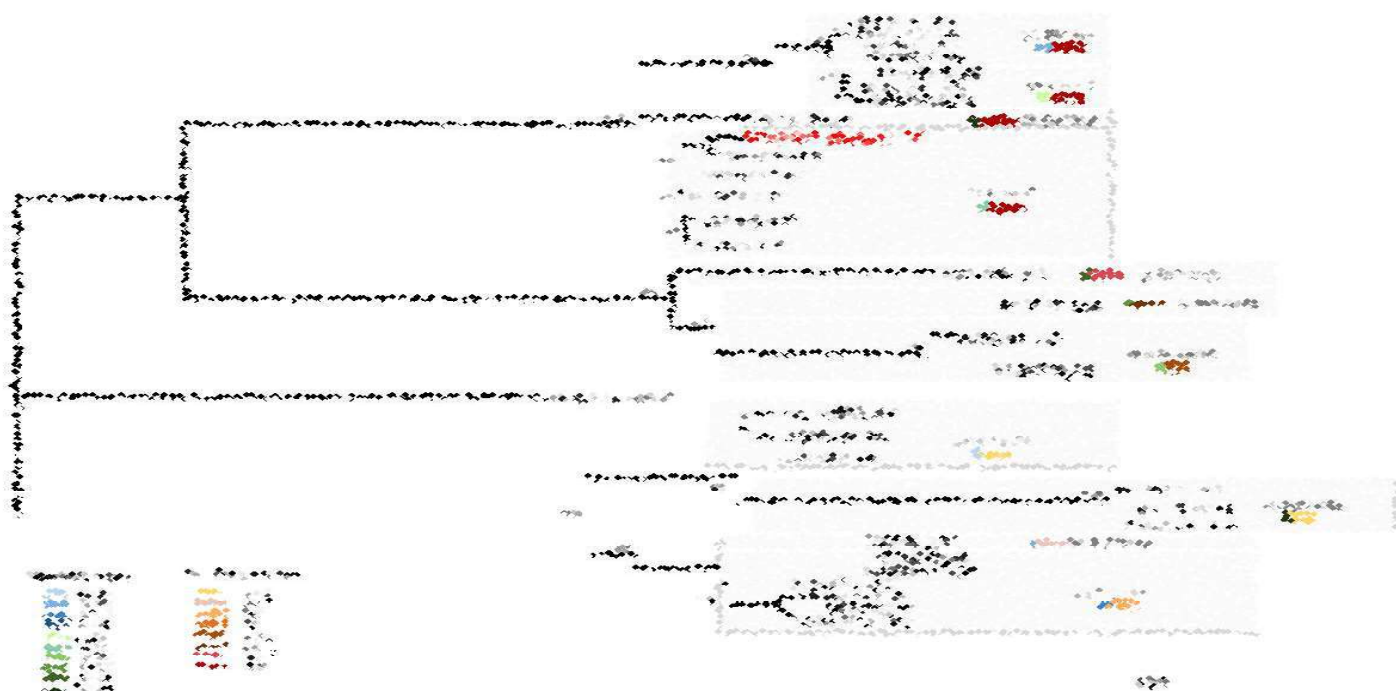
Chapter 3

Research papers on RHDV2

Chapter 3 includes three research papers on RHDV2, namely a study of a potential wild species reservoir, a case report of a GI4P-GI.2 detection on a vaccinated pet rabbit and the description of the first cases of co-infection by RHDV2 and myxoma virus
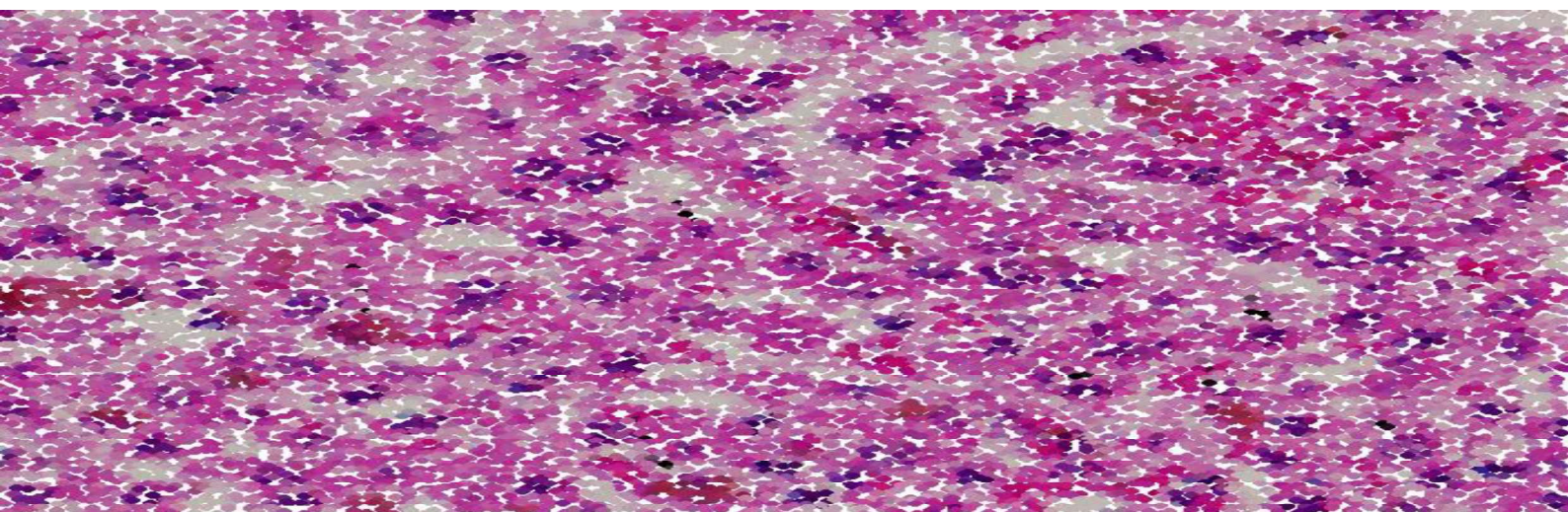
What is the importance of wild rabbit sympatric species in the epidemiology of RHD?

Scientific publication 6

Spillover events of rabbit haemorrhagic disease virus 2 (recombinant GI.4P-GI.2) from Lagomorpha to Eurasian badger

■ ■

Reservoirs are key in the persistence of virus sources. The reservoir specie for RHDV/RHDV2 are unkown. European badger was identified as a possible reservoir host of RHDV2.

ORIGINAL ARTICLE

Transboundary and Emerging Diseases **WILEY**

Spillover events of rabbit haemorrhagic disease virus 2 (recombinant GI.4P-GI.2) from Lagomorpha to Eurasian badger

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Abstract

Rabbit haemorrhagic disease (RHD) is a major threat to domestic and wild European rabbits. Presently, in Europe, the disease is caused mainly by Rabbit haemorrhagic disease virus 2 (RHDV2/b or *Lagovirus europaeus* GI.2), the origin of which is still unclear, as no RHDV2 reservoir hosts were identified. After the RHDV2 emergence in 2010, viral RNA was detected in a few rodent species. Furthermore, RHDV2 was found to cause disease in some hare species resembling the disease in rabbits, evidencing the ability of the virus to cross the species barrier. In this study, through molecular, histopathologic, antigenic and morphological evidences, we demonstrate the presence and replication of RHDV2 in Eurasian badgers (*Meles meles*) found dead in the district of Santarém, Portugal, between March 2017 and January 2020. In these animals, we further classify the RHDV2 as a *Lagovirus europaeus* recombinant GI.4P-GI.2. Our results indicate that *Meles meles* is susceptible to RHDV2, developing systemic infection, and excreting the virus in the faeces. Given the high viral loads seen in several organs and matrices, we believe that transmission to the wild rabbit is likely. Furthermore, transmission electron microscopy data show the presence of calicivirus compatible virions in the nucleus of hepatocytes, which constitutes a paradigm shift for caliciviruses' replication cycle.

KEYWORDS

Eurasian badger, GI.2, *Meles meles*, recombinant GI.4P-GI.2, RHDV2/b, spillover event

1 | INTRODUCTION

Rabbit haemorrhagic disease virus 2 (RHDV2, also referred to as *Lagovirus europaeus* GI.2 or RHDVb (Le Pendu et al., 2017)) is a highly contagious Lagovirus of the Caliciviridae family that causes an acute infection in wild and domestic European rabbits (*Oryctolagus cuniculus*).

The virion measures from 27 to 40 nm (Capucci et al., 1991; Ohlinger et al., 1990) in diameter with the icosahedral typical morphology of the Caliciviridae and a surface consisting of regularly arranged, cup-shaped depressions (Capucci et al., 1991; Ohlinger et al., 1990). The genomic material is composed of a single-stranded, non-segmented, positive-sense RNA genome of 7,437 nucleotides (Meyers et al., 1991). Also, the virus contain a sub-genomic 2.2 kb RNA, collinear to the 3′ region of the genome (Ohlinger et al., 1990).

RHDV2 emerged in 2010 in France (Le Gall-Recule et al., 2011) and rapidly replaced the former circulating RHDV classical strains (GI.1) in several European countries, also expanding to many regions in the world (Mahar et al., 2018). Since 2012, no RHDV GI.1 strains (former G1 to G6) have been reported in the Iberian Peninsula. Furthermore, following the detection of RHDV2 strains, recombinant strains containing the non-structural region of GI.1 or non-pathogenic RHDV strains (GI.3 and GI.4) and the structural region of RHDV2 (GI.2) strains were identified. Several recombination breaking points have been described (Silvério et al., 2018). Of these, the recombinant hotspot located at the RdRp/VP60 level seems to play an important role in the evolution of the new variants (Abrantes et al., 2020; Dalton et al., 2018; Silvério et al., 2018).

Another member of the Lagovirus genus, the rabbit calicivirus (RCV, GI.5; Capucci et al., 1996) is an asymptomatic virus of the intestinal tract. In addition to these, the European RCV-E1 (GI.3) and Australian RCV-A1 (GI.4; Le Gall-Reculé et al., 2011; Strive et al., 2009) are non-pathogenic rabbit viruses. The information on these viruses is scarce but can contribute as genetic donors resulting in recombinant pathogenic viruses (Silvério et al., 2018).

Other than in the domestic and wild rabbit, RHDV2 has also been reported in several hare species, namely the Sardinian Cape hare (*Lepus capensis mediterraneus*; Puggioni et al., 2013), the Italian hare (*Lepus corsicanus*; Camarda et al., 2014), the European brown hare (*Lepus europaeus*; Velarde et al., 2017) and the Mountain hare (*Lepus timidus*; Neimanis et al., 2018) and recently in the jackrabbit (*Lepus californicus*; USDA, 2020), desert cottontails (*Sylvilagus audubonii*; USDA, 2020) and eastern cottontail (*Sylvilagus floridanus*; USDA, 2020), causing haemorrhagic disease similar to that of the European rabbit.

The concumitant ability of RHDV2 to infect hares, alongside with the high fatality rates in both young and adult rabbits (Le Gall-Recule et al., 2011), contrasts the epidemiological and pathological pattern of RHDV GI.1, which affects only rabbits older than 4 weeks of age (Liu et al., 1984), although there is isolated evidence of RHDV-RNA presence in hares (Lopes et al., 2014).

In areas where RHDV2 circulates, sympatric Iberian meso-carnivores come in contact with the virus, either by predation or necrophagy of infected leporids or through contact with contaminated soil or vegetation (OIE, 2019). RHDV2-RNA was identified in pine voles (*Microtus pinetorum*) and white-toothed shrews (*Crocidura russula*), two rodent species that are sympatric to the European wild rabbit (Calvete et al., 2019). The RHDV2 transmission to small mammals could happen due to their scavenging habits, or by ingestion of rabbit infected tissues or faeces (Calvete et al., 2019). Additionally, it is also known that red foxes (*Vulpes vulpes*; Chiari et al., 2016) and wolf (*Canis lupus*; Di Profio et al., 2017) play a role as passive carriers of lagoviruses, spreading European brown hare syndrome virus (EBHSV) by their faeces after ingestion of infected hares.

Given this possibility, several sympatric species of wild animals have been investigated for RHDV2 at our laboratory over the last three years (data not shown), namely birds, rodents and carnivores, including mustelids such as the Eurasian badger. The Eurasian badger (*Meles meles*) is a gregarious, fossorial, and evasive species. Badgers are predominantly nocturnal or crepuscular (Fedriani et al., 1999), mainly in the spring, when the wild rabbit activity peak coincides with the peak of RHD (Mutze et al., 2002). This trophic, opportunistic, generalist species preys on the European rabbit, particularly juvenile (Fedriani et al., 1999). In the summer and autumn/winter, lagomorphs represent about 60% and 22.7% of a badger's diet, respectively (Fedriani et al., 1999), whereas in the spring rabbit may account for up to 87.9% of the ingested biomass according to a study carried out in Spain (Fedriani et al., 1999).

This study was part of a three years monitoring programme to investigate haemorrhagic disease in the wild rabbits and sympatric species collected in mainland Portugal. Among several non-leporid species investigated, some of the badgers tested RHDV2 positive by molecular methods. This finding prompted us to further investigate the presence of RHDV2 proteins, viral particles and RHDV2 compatible lesions in this species. To our knowledge, we are reporting for the first time the *Lagovirus europaeus* GI.4P-GI.2 crossing the barrier beyond the Lagomorpha order, namely to the Eurasian badger.

2 | MATERIALS AND METHODS

2.1 | Origin of the animals

A total of 10 European badgers were admitted for this study. All the information collected about these animals, namely sex, weight and date of collection, among others, is summarized in Table S1.

One badger (B1) found dead on the road and nine others (B2 to B10) found dead in agricultural areas were collected during active prospection for wild rabbit carcasses and sympatric species, namely foxes, insects, birds and rodents, within the scope of an epidemiological evaluation of haemorrhagic disease in rabbit populations. All badger carcasses were found in the District of Santarém (Portugal NUT III, PT185 area).

The poor quality of the wildlife biological samples posed a major challenge to the laboratory diagnosis, which was overcome through the establishment of a comprehensive diagnosis strategy resourcing

to several molecular, antigen detection methods, sequencing and transmission electron microscopy (TEM). Thus, the methodological strategy adopted was as follows: samples were tested by RT-qPCR (Duarte et al., 2015) and the negatives were excluded from further analyses. The RT-qPCR-positive badgers showing milder autolysis were selected for RHDV2 sequencing analysis, immunohistochemistry (IHC) and TEM.

2.2 | Necropsy sampling and histopathology

B1 was maintained at −20°C until necropsy, while the remaining carcasses were kept at 4°C for a maximum period of 2 days. No badger was necropsied along with other species, namely rabbits, to avoid cross- contaminations. Necropsies were performed according to the routine implemented practices, as described in the necropsy manual (Peleteiro et al., 2016), and carried out at the Faculty of Veterinary Medicine, University of Lisbon (FMV-ULisboa, Lisbon, Portugal) or at the National Institute for Agricultural and Veterinary Research (INIAV I.P., Oeiras, Portugal).

Different samples were collected for molecular diagnosis, histopathology and bacteriology. To avoid faecal contamination, the sample collection was carried out in the following order: liver, spleen, kidney, lung, left ventricle, other organs, duodenum and finally faeces. Disposable material was used for each organ to avoid cross-contamination between matrices. Blood samples were not collected due to coagulation and/or autolysis.

For histopathology, liver, spleen, duodenum, stomach, kidney, lung, left ventricle, trachea and mesenteric lymph nodes were collected and fixated in 10% (v/v) neutral buffered formalin, routinely paraffin-embedded, sectioned at 3 μm and stained with haematoxylin and eosin (H&E). Microphotographs were obtained with a DP23 Olympus digital camera.

Perls Prussian Blue was used to detect the presence of non-haem iron (ferritin and haemosiderin) in the tissues (Bancroft & Stevens, 1990).

2.3 | Immunohistochemistry and dot blotting

Immunohistochemistry was performed as follows: (a) 4 μm sections of liver samples were mounted in appropriated glass slides; (b) deparaffinization and antigen retrieval were performed in a PT Link (Dako) at 96°C for 20 min, with low pH EnVision FLEX target retrieval solution (code DM829; Dako); (c) peroxidase block (with Peroxidase blocking solution, DAKO) was carried out for 20 min at room temperature; (d) primary antibody (named pAb or Virlab derived from a hyperimmune serum collected from a rabbit infected in the facilities of the University of Oviedo (UniOvi) with strain RHDV-Ast89 (RHDV)), diluted at the optimized concentration (1/1000) in Antibody Diluent (DAKO), was incubated for 1 hr at room temperature; (e) incubation with the secondary antibody (at the concentration indicated by the manufacturer (Dako EnVision™+ Dual Link System-HRP, mouse

anti-rabbit)), for 30 min at room temperature; (f) staining with DAB chromogen (DAKO) for 5 min; (g) nuclei counterstain with Gills haematoxylin and finally (h) dehydration in an ethanol gradient. Finally, the sections were mounted with a non-aqueous mounting medium. The necessary washes between incubations were performed with phosphate-buffered saline, pH 7.4 (PBS) twice for 5 min each with frequent manual agitation. The pAb was previously validated against RHDV and RHDV2 (González, 2019).

Dot blot was carried out using approximately 20% (w/v) homogenates prepared in PBS of liver and lung samples from badgers B5 and B6 after clarification at 6,000 g for 5 min. Liver homogenates from RHDV2-positive and RHDV2-negative rabbits were used as positive and negative controls, respectively. A grid was drawn on a piece of nitrocellulose membrane (Thermo Fisher Scientific Inc.) using a pencil. 1 μl drops were deposited in the middle of the squares, and the membrane was allowed to dry for 15 to 20 min at room temperature. The inhibition of non-specific reactions was performed by shaking incubation with 5% milk powder in PBS (w/v) for 45 min at room temperature. Two 5-min washes with shaking were performed with 0.05% Tween 20 (v/v) in PBS. Then, the membranes were incubated for 1h at room temperature with agitation with primary antibodies diluted at the optimized concentrations in 0.05% Tween 20 in PBS. Two monoclonal antibodies (mAb) were used, namely 1A2 (anti-VP60, Ingenasa) and 3A10 (anti-VP60 of RHDV and RHDV2, S domain, produced in the Department of Biochemistry and Molecular Biology (UniOvi)). The polyclonal antibody (pAb) was also used. After incubation, two additional washes were performed as described above. The membranes were then incubated in the dark under shaking conditions with the secondary antibody, goat anti-rabbit IgG, conjugated with fluorescein isothiocyanate (FITC; Merck Life Science S.L.U. suc) for the polyclonal serum or anti-Mouse IgG (whole molecule)—FITC antibody produced in goat (Sigma-Aldrich) for the monoclonal antibodies, both diluted at 1:200 in PBS-Tween (0.05% Tween 20 (v/v) in PBS) for 1 hr, at room temperature. Finally, after two wash steps of 5 min each, visualization of membranes was carried out using Odyssey Infrared Imaging System (LI-COR Biosciences) and the software Image Studio (LI-COR Inc.).

2.4 | Immunofluorescence

Tissue samples from B5 and B6 were embedded in optimal cutting temperature (OCT) compound, and sections were cut onto slides using a cryostat at −20°C. The cryostat sections were permeabilized using 0.2% saponin in PBS (w/v) for 20 min before emerged in blocking solution (0.02% saponin, 1% BSA in PBS) for 30 min at room temperature. Antibody labelling was performed by incubating sections with pAb (polyclonal antibodies) and the mouse monoclonal anti-LAMP1 antibody (ab25630, Abcam) in blocking solution for 2 hr at room temperature. Secondary antibodies used at 1:500 (Goat anti-Rabbit IgG (H + L) cross-adsorbed secondary antibody, Alexa Fluor 488 (ThermoFisher A-11008) and goat anti-mouse IgG (H + L) cross-adsorbed secondary antibody, Alexa

Fluor 555 (ThermoFisher A-21422)) and in some cases, fluorescent phalloidin (ab176759, Abcam), were added to the sections and incubated for 1 hr at room temperature. Finally, coverslips were added to the slides using mounting media containing DAPI (ab228549, Thermo Fisher Scientific Inc.). Images were acquired in a Leica SP8 confocal microscope, and greyscale inverted images were produced using ImageJ (NIH). Negative controls were either uninfected or infected samples where the polyclonal antibody was omitted.

2.5 | Molecular diagnosis of RHDV, sequencing and phylogenetic analysis

The tissue and faeces samples were homogenized at 20% in sterile PBS (w/v), using mechanical homogenization with 0.5 mm zirconium beads (Sigma-Aldrich) and the FastPrep FP120 Bio101 Homogenizer (Level 5 velocity, 45s, Savant Instruments). Total nucleic acids were extracted from 200 µl supernatant after a 5 min 6,000g centrifugation. The automatic extraction was performed with the MagAttract 96 cador Pathogen kit (Qiagen) on the BioSprint 96 automatic extractor, following the protocol supplied with the kit.

Each tissue and faecal sample was tested for RHDV2-RNA (GI.2) by a specific RT-qPCR developed by Duarte et al. (2015) that targets a 127 nt region of the VP60 gene, using the OneStep RT-PCR Kit (Qiagen).

cDNA was synthesized with the SuperScript™ IV First-Strand Synthesis System (InVitrogen) according to the manufacturer's recommendations, using either oligo(dT)$_{12-18}$ and random hexamers.

The conventional RT-PCR (Dalton et al., 2018) for differentiation of RHDV2 strains from RHDV2 recombinants was carried out using the Phusion Flash High-Fidelity PCR Master Mix (Thermo Fisher Scientific Inc.) according to the manufacturer's recommendations.

For two positive liver samples (B5 and B6), amplification of the partial RdRp gene and full VP1 (VP60) gene sequence was accomplished using the primers described in Table S2, where all the primers used in this investigation are shown. Regarding the other samples, partial sequencing (Table S1) was performed to confirm the presence of RHDV2.

The amplicons were purified using the NZY GelPure kit (NZYTech), following the manufacturer's instructions, and directly sequenced with a 3130 Genetic Analyser (Applied Biosystems). The resulting sequences were analysed using Seqscape software v2.7 (Applied Biosystems). Merged and assembled consensus sequences were submitted to GenBank.

The partial sequence of RNA polymerase (RdRp) gene and the complete vp60 gene sequence from badger B6 were compared with reference strains for all known RHDV and RHDV2 genotypes, and variants that infect the European rabbit for which this genomic region are available in the database. The accession numbers are shown in the phylogenetic tree (Figure 16). The alignment was achieved using the Mega X, for a total of 2,191 positions (partial RdRp gene and complete VP60 gene) and the evolutionary history inferred using the Maximum Likelihood method and General Time Reversible model (Nei & Kumar, 2000). The model was selected using the Mega Function having selected the model GTR + G+I (BIC 37184.06 and AIC 36028.07).

The known principal genotypes and variants of *Lagovirus* that can infect the *Oryctolagus cuniculus* are shown in the figure, as reference. The tree with the highest log likelihood (−17929.21) is shown.

2.6 | Transmission electron microscopy

Transmission electron microscopy (TEM) was performed in liver samples from three badgers (B1, B5 and B6). Briefly, samples were fixed for 72 hr with a solution 0.1 M sodium cacodylate (Sigma-Aldrich) containing 2.5% glutaraldehyde (Sigma-Aldrich). The regions of interest were sliced in small ~1 mm^3 and washed five times in 0.1 M sodium cacodylate buffer, and samples were then post-fixed with 1% osmium tetroxide (EMS) for 1 hr, and en bloc stained with 1% Millipore-filtered uranyl acetate (Agar Scientific). Samples were dehydrated in increasing concentrations of ethanol, infiltrated and embedded in EPON 812 (EMS). Polymerization was performed at 60°C for 24 hr, and ultrathin sections were obtained in a Reichert ultracut E ultramicrotome (Leica), collected to 1% formvar-coated copper slot grids (Agar Scientific), stained with 2% aqueous uranyl acetate and lead citrate and examined in a Jeol 1400 plus transmission electron microscope at an accelerating voltage of 120 kV. Digital images were obtained using an AMT XR16 bottom mid mount digital camera (AMT). The sections were systematically analysed using AMT© software and several high and low magnifications were acquired.

2.7 | Other virological analyses

Tissue and faeces samples were homogenized and total nucleic acids extracted as described above. Feline and canine Parvoviruses, canine distemper virus (morbillivirus), mammal (canine, feline and porcine) coronaviruses, type A rotavirus, Ausjesky disease virus and other herpesviruses were investigated using the methods and matrixes described in Table S3.

2.8 | Bacteriological analysis

Liver, spleen, kidney and lung from B5, B6, B7 and B8 badgers were collected at necropsy for bacteriology using conventional techniques. In the case of B5 and B6, all samples were processed separately. A pool of organs from the animal was prepared for B7 and B8. The remaining badgers were not sampled for bacteriology due to extensive autolysis or faecal contamination.

Briefly, aerobic bacteria isolation was performed on Columbia agar supplemented with 5% sheep blood (BioMérieux) and MacConkey agar (Thermo Fisher Scientific Inc.), incubated at 37°C for 24 to 48 hr. Strict anaerobic bacteria isolation was performed on Schaedler agar supplemented with 5% sheep blood (BioMérieux) and incubated for 48 hr at 37°C in the absence of oxygen. Afterwards, isolates were identified through their macro- and microscopic morphology, staining characteristics and biochemical profiling using API (BioMérieux) galleries, according to the manufacturer's instructions.

2.9 | Parasitological analysis

Faeces were collected for the flotation method to detect helminth eggs or protozoan oocysts. Faeces were mixed with saturated sugar solution (1:4), and after pouring the solution into tubes, lamellae were placed on their top to allow adhesion to the glass and allowed to stand for 15 min. Lamellae were then transferred to slides, and preparations were observed with a compound microscope.

Gastrointestinal content was also collected and placed on a tray with a black background, filled with warm water and, after standing for a few minutes, supernatant was removed by decantation. This procedure was repeated three times until the liquid became clear enough to visualize the presence of helminths by contrast. Furthermore, flotation method for egg and oocyst detection was also performed as described above.

3 | RESULTS

3.1 | Necropsy data

Necropsy of badger B1 revealed a good overall body condition, multiple fractures of the skull and lacerations in the liver and spleen, interpreted as resulting from a traffic accident. The autolysis limited the interest in the identification of the lesions, both grossly and microscopically. B2, B3, B4, B9 and B10 presented severe autolysis, good body condition and no visible lesions. Badgers B5 and B6 were emaciated, severely dehydrated, with uncoagulated blood, congestive foci in the lungs and slightly discolored liver. B7 and B8 presented similar lesions, however with coagulated blood. Other data from the necropsies are shown in Table S1 available as supplementary data.

3.2 | Histopathological findings

Histopathology data for badgers B5 to B8 are summarized in Table 1, and the main findings shown in Figures 1 to 10.

Liver of 3 out of 4 badgers showed areas of hepatocellular hydropic degeneration and individual cell necrosis (Figures 1 and 2). In two cases (B7 and B8), generalized moderate oedema of the perisinusoidal space (space of Disse) was evident. Kupffer cells containing haemosiderin were identified in B8.

Spleen showed haemosiderin–laden macrophages in the red pulp in most cases. This was marked in badgers B5 and B8, posteriorly demonstrated with Perls Blue stain for iron (Figure 3) and mild to moderate in all others. In two badgers (B5 and B6), white pulp hyperplasia was also seen (Figure 4). In one case (B8), not coincident with the lymphoid hyperplasia, focal deposits of acidophilic material (fibrin-like) were present in germinal centres (Figure 5).

In the lungs, severe congestion of interalveolar septa was present in badger B6 and B7 (Figure 6). Small aggregates of haemosiderin-containing macrophages were detected in the interalveolar septa in one case (B6) (Figure 7). In two badgers, interalveolar septa were thickened due to marked to moderate infiltration of mononuclear inflammatory cells (diffuse interstitial pneumonia; Figure 8).

In the kidneys, vacuolar and granular degeneration of tubular cells in the proximal and distal tubules was present in all cases. In some of these tubules, necrosis of tubular cells was evident. In the heart, cardiomyocyte degeneration consisting of loss of striation and granular appearance of the cytoplasm was evident (Figure 9). In one case (B6), this was accompanied by focal haemorrhage (Figure 10).

3.3 | Immunohistochemistry and Dot blot

To investigate the presence of the virus in the tissues, detection of the viral capsid protein (mainly composed by VP60) was attempted in badger and rabbit tissues by immunolabelling techniques using polyclonal (pAb) against RHDV and monoclonal antibodies (1A2 and 3A10).

Immunohistochemistry of badgers' livers carried out with the pAb allowed the observation of generalized cytoplasmic staining of the hepatocytes of badgers B5 (Figure 11) and B6 (showing similar staining, result not shown), contrarily to the absence of staining in liver samples from a qPCR-negative badger and a qPCR-negative rabbit (results not shown). In addition to cytoplasmic staining in badgers B5 and B6, some hepatocytes also showed nuclear staining.

The same positive reaction for badgers B5 (weaker) and B6 (stronger) was observed in the dot blot analyses (Figure 12). Like in immunohistochemistry, in the dot blot, the pAb generated stronger reactions.

3.4 | Immunofluorescence

To further demonstrate replication of RHDV2, indirect immunofluorescence was performed in the liver of badgers B5 and B6 (named #1 and #2, respectively, in Figure 13). We observed encapsulated virions, recognizable by the FITC antibody directed to VP60, whose density is compatible with active replication of RHDV2 in the tissues (Figure 13). DAPI staining was used for labelling DNA in fluorescence microscopy and phalloidin to stain the actin filaments. Livers from an RHDV2-infected rabbit and the RHDV2 non-infected badger were used as a positive and negative control, respectively.

TABLE 1 Histopathological findings in badgers B5, B6, B7 and B8

ID	Liver	Spleen	Lung	Kidney	Others
B5	Hepatocytes with minimal changes. In some areas, they show mild hydropic degeneration.	Accumulation of haemosiderin–laden macrophages in the red pulp. Mild hyperplasia of lymphoid follicles, which show foci of acidophilic material compatible with fibrin in the germinal centres.	Thickening of interalveolar septa with infiltration by mononuclear inflammatory cells (diffuse interstitial pneumonia). Frequent aggregates of haemosiderin–laden macrophages. Vicarious alveolar emphysema. Shrinked pleura.	Vacuolar and granular degeneration of epithelial cells in the proximal and distal tubules.	Cardiomyocytes granular degeneration and loss of striation. Thymus: necrosis of 10%–15% of lymphocytes, which show karyolysis and karyorrhexis.
B6	Severe congestion. Hepatocytes with generalized and severe hydropic degeneration. About 50% of the hepatocytes show no nucleus (karyolysis).	Hyperplasia of lymphoid follicles. Diffuse infiltration of red pulp by mononuclear cells (chronic splenitis).	Severe congestion and moderate thickening of alveolar septa by mononuclear inflammatory cells (interstitial pneumonia). Frequent aggregates of haemosiderin–laden macrophages. Vicarious alveolar emphysema.	Congestion of the cortex. Necrosis of tubular epithelium of proximal and distal tubules.	Cardiomyocytes granular degeneration and loss of striation. Haemorrhagic foci.
B7	Occasional hyperchromatosis of the nuclear membrane, karyorrhexis and pycnotic nuclei. Space of Disse oedema with mild atrophy of hepatocytes.	Lymphoid follicles hypoplasia and fibrotic changes of the red pulp (chronic splenitis).	Severe congestion and moderate thickening of alveolar septa by mononuclear inflammatory cells (interstitial pneumonia). Vicarious alveolar emphysema.	Vacuolar and granular degeneration of epithelial cells in the proximal and distal tubules.	Cardiomyocytes granular degeneration and loss of striation.
B8	Generalized hepatocyte hydropic degeneration. Occasional hyperchromatosis of the nuclear membrane, karyorrhexis and pycnotic nuclei. Intralobular Kupffer cells containing haemosiderin. Space of Disse oedema.	Accumulation of haemosiderin–laden macrophages in the red pulp. Lymphoid follicles show small deposits of acidophilic material compatible with fibrin in the germinal centres together with haemosiderin–laden macrophages.	Thickening of interalveolar septa with infiltration by mononuclear inflammatory cells (diffuse interstitial pneumonia).	Vacuolar and granular degeneration of epithelial cells in the proximal and distal tubules.	Cardiomyocytes granular degeneration and loss of striation.
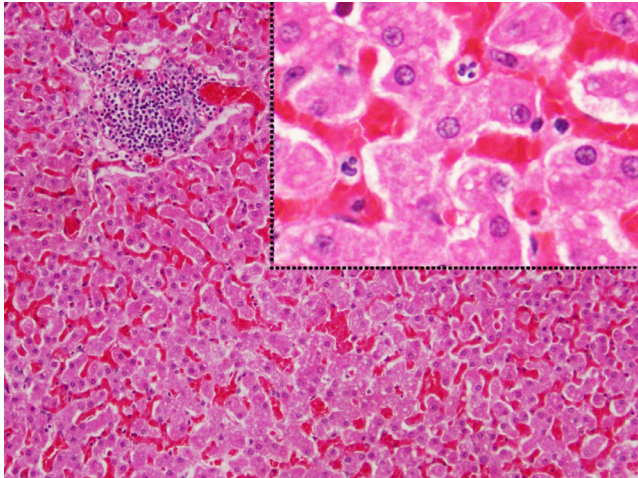
FIGURE 1 Liver (B6). Mild hydropic degeneration of the hepatocytes in the lower centre. Focal infiltration of mononuclear cells in the upper left. The hydropic degeneration can be seen at higher magnification in the inset. H&E, ×100, inset, ×400

FIGURE 2 Liver (B8). Hydropic degeneration of the hepatocytes. Some cells show pyknosis and karyorrhexis (short arrows), as well as hyperchromatosis of the nuclear membrane; oedema of the perisinusoidal space and Kupffer cells containing haemosiderin (long arrows). H&E, ×400

FIGURE 3 Spleen (B8). Marked presence in the red pulp of haemosiderin containing macrophages. Perls Blue, ×40, inset ×100
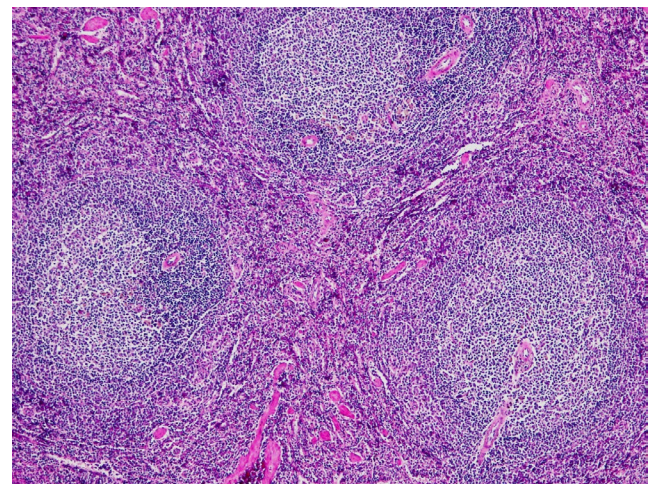
FIGURE 4 Spleen (B6). Hyperplasia of the lymphoid follicles. H&E, ×100

FIGURE 5 Spleen (B8). The centre of some lymphoid follicles shows focal deposits of acidophilic material (*). H&E, ×40, inset ×100

A clear green labelling is observed in both badgers' livers and in the RHDV-2 positive rabbit sample. The badgers' samples showed distinct punctate staining (more evident in the B6), ascribing virus localization to subcellular compartments. Due to the poor tissue preservation conditions, DAPI is dispersed by the cytoplasm of cells. For the same reason, phalloidin is sometimes also difficult to observe.

To evaluate the presence and increment of lysosomal or autophagosome-like structures that are usually associated with viral factories, LAMP 1 fluorescence staining was performed. The membrane glycoprotein 1 was detected by direct immunofluorescence (Figure 14a) in the cytoplasm of infected hepatocytes. The presence of a large number of lysosomes was also confirmed in hepatocytes

FIGURE 6 Lung (B6). Severe diffuse congestion of the alveolar septa some of which are thickened due to inflammatory cell infiltration. Granuloma in the lower right. H&E, ×40
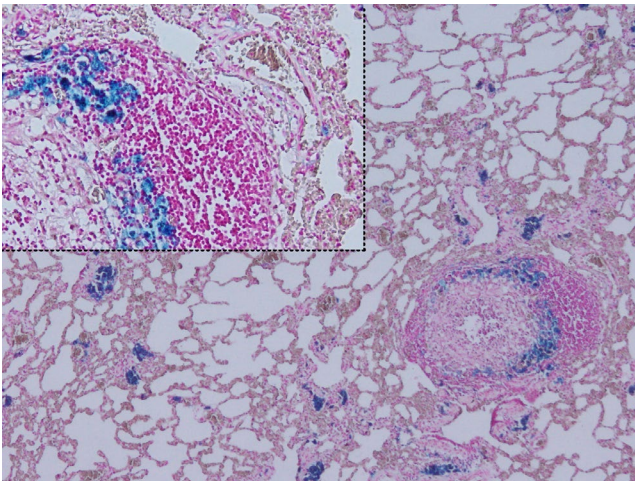
FIGURE 7 Lung (B6). Iron deposits in the alveolar septa and in macrophages in the periphery of a granuloma. Perls Blue, ×40, inset ×100
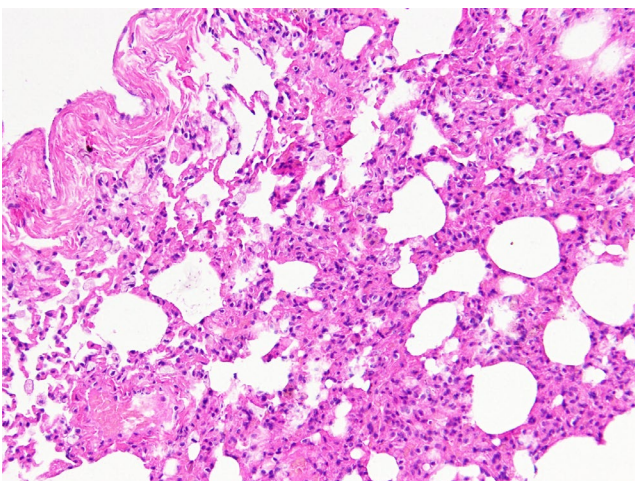
FIGURE 8 Lung (B8). Diffuse interstitial pneumonia. The pleura is corrugated. H&E, ×100
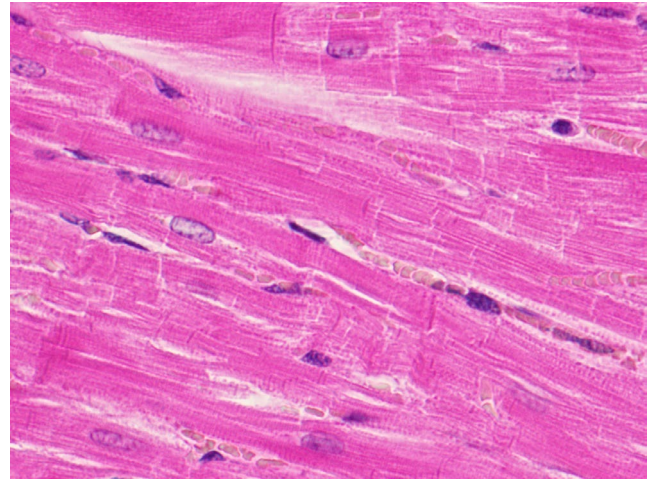
FIGURE 9 Myocardium (B5). Striation loss and granular appearance of the cardiomyocites. H&E, ×400
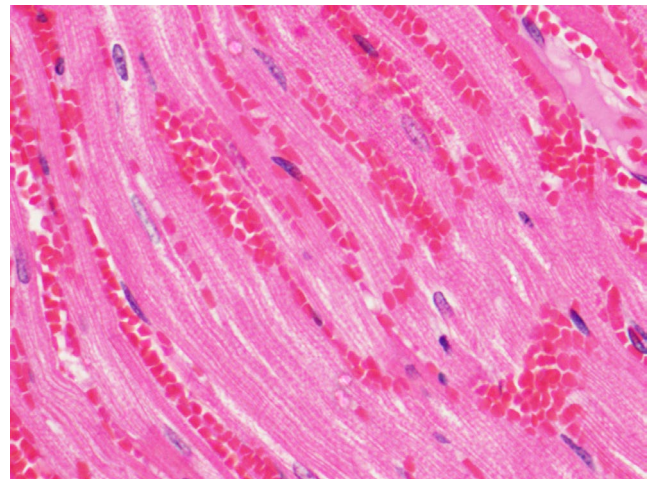
FIGURE 10 Myocardium (B6). Focal intercellular haemorrhage. Striation loss and granular appearance of the cardiomyocites. H&E, ×400

of RHDV2-infected badgers (B1, B5 and B6) by TEM showing that viral particles colocalize with lysosomal structures, indicating that the virus is within lysosomes (Figure 14b,c).

3.5 | Molecular diagnosis and sequencing analysis

RHDV2-RNA was detected by RT-qPCR in most of the organs from badgers B1, B5–B10 (Table 2), suggesting that the virus was disseminated systemically. In general, the higher viral loads were observed in the liver and spleen (Table 2). None of the badgers tested positive for RHDV GI.1-RNA.

Full RHDV2 vp60 sequences were obtained for badgers B5 and B6 showing these to be RHDV2 strains (GI.2). Despite RHDV2 vp60 genes from badgers B1, B7 to B10 vp60 genes not being sequenced, given the specificity of the RT-qPCR method used for
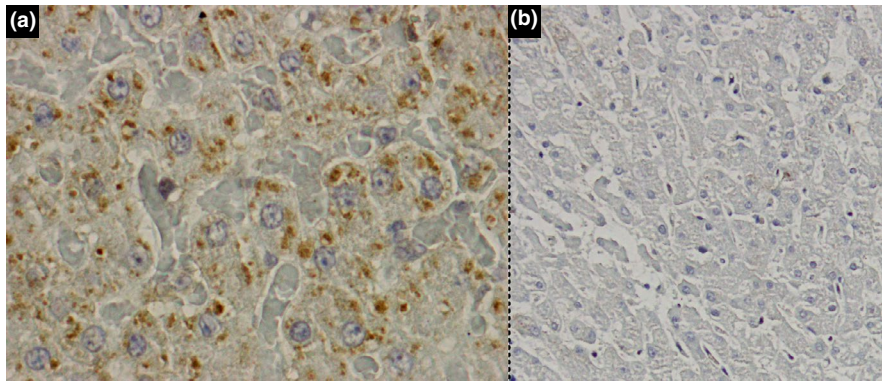
FIGURE 11 Immunostaining for RHDV2 in the liver of badger B5 (left). Hepatocytes show cytoplasmic granular brown staining. At right, negative control of a non-infected badger liver. DAB counterstained with Gills haematoxylin, ×400
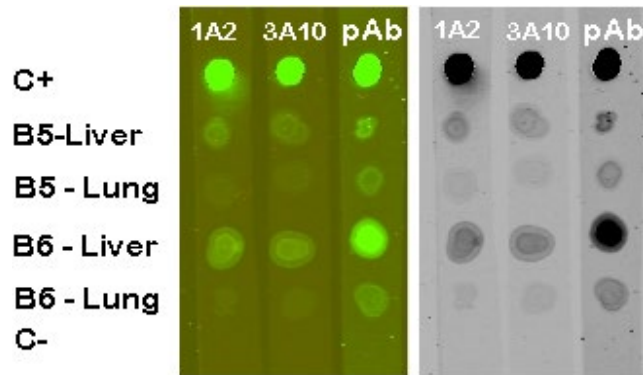
FIGURE 12 Dot blots from B5 and B6 badgers. C + homogenate from a RHDV2-positive rabbit. C - Bovine serum albumin

RHDV2 (Duarte el al., 2015) detection to which they were positive, allowed to conclud that the strains from these badgers were also GI.2.

To further investigate the genomic composition of the RHDV2 badgers' strains, an upstream genomic region comprising the 3′ end of the RdRp gene was amplified by a conventional RT-PCR (Dalton et al., 2018) and sequenced.

The partial RdRp nucleotide sequences from badgers B1(MW446907), B5(MT610362), B6(MT610363), B7(MW446908), B8(MW446909) and B9(MW446910) showed higher identity to GI.4 strains regarding this non-structural gene.

The final RdRp/VP60 2,188 nucleotide sequences obtained from badger B5 (MT610362) and badger B6 (MT610363) were aligned and compared, showing 100% identity with each other. BLAST search (performed on 5th January 2021) using the VP60 sequence showed higher similarities, namely 99.37% and 98.10% with MG763954 (a recombinant strain reported in 2018 from a wild rabbit collected in Estremoz, south Portugal), and KF442963 (also a recombinant strain obtained in 2013 from a wild rabbit from in Barrancos, south Portugal), respectively. The BLAST analysis of the RdRp partial sequences of B5 and B6 showed higher identity with the same sequences, namely 97.54% with sequence MG763954.

The partial RdRp nucleotide sequences (409nt long) from badgers B5 and B6 shared 95% of identity with B1, 83% with B7 and B8, and 82% of identity with B9.

The amino acid sequence of the partial RdRp (149 residues long) deduced from sequence MT610363 (B5) showed 98.66% identity

with the homologous region from sequence AIT40572.2 (RHDV2 collected from a wild rabbit from Estoi, Faro, south of Portugal in 2013). Also, the complete amino acid sequence of VP60 gene (579 residues long) deduced from sequence MT610363 showed 99.83% identity with the VP60 sequence from a strain collected from Valpaços, Vila Real Portugal in 2012 (AJE29738).

When the deduced amino acid (aa) sequences comprising the contiguous partial RdRp gene and the complete VP60 gene were compared with the sequences available in the GenBank database, the greatest identity (99.59%) was found with sequence AIT40572.2. With regards to this sequence, three aa substitutions were observed in the RHDV2 badger sequence MT610363, namely an Ala^{1648} to Thr^{1648} (a radical aa residue change) and a Tyr^{1737} to His^{1737} (conservative replacement) located in the RdRp protein, and one substitution in the VP60 protein, namely a Thr^{2109} to Ser^{2109} (conservative replacement), located in loop L2 of P2 sub-domain (positions indicated refer to the polyprotein). These three aa residues found in the B5 and B6 RHDV2 polyprotein are not unique in badgers' strains, being also found in rabbits' strains.

3.6 | Phylogeny of the badgers' RHDV2 strains

The 2,191-nt long region containing the partial RdRp gene and full VP1 (VP60) gene sequences from badger B5 (MT610362) and B6 (MT610363) was used to assess the phylogenetic relationships between these strains and the RHDV and RHDV2 strains from wild rabbits by maximum likelihood (ML; Figure 15).

As expected, the RHDV2 badger strains, represented by sequence MT610363, were grouped with Portuguese recombinant RHDV2 strains, close to the reference strain RCV-A1-like/RHDV2 (MG763954), with variant of GI.4 of polymerase gene and GI.2 VP60 genotype. The access numbers of the sequences included in the tree are shown in the phylogenetic tree (Figure 15).

3.7 | Transmission Electron Microscopy

Viral particles were identified by transmission electron microscopy (TEM) in liver of badgers B1, B5 and B6. Virions showed the typical icosahedral morphology and size was of 33.4 ± 4.1 nm (mean ± SE,
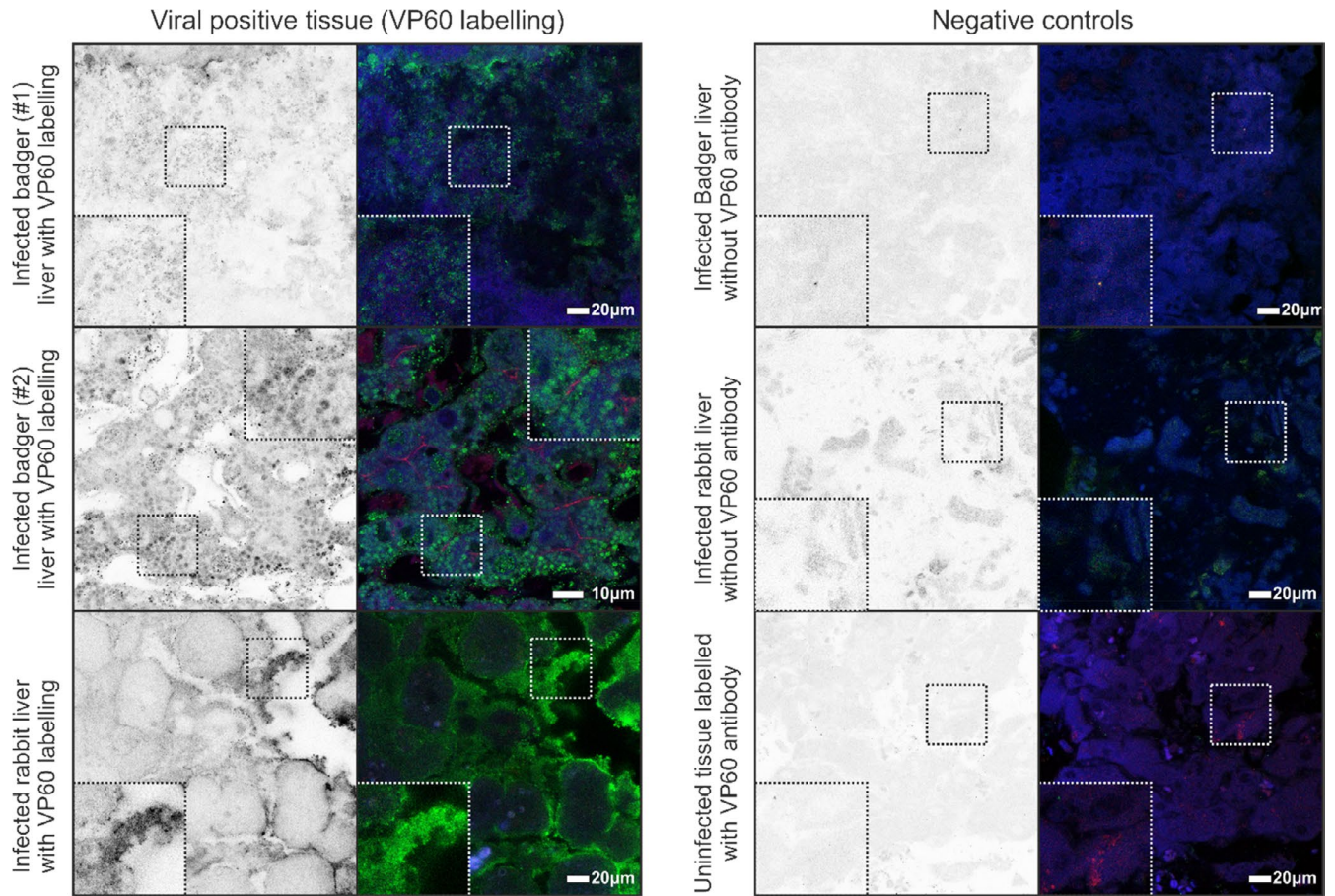
FIGURE 13 Immunofluorescence using Virlab polyclonal antibody to detect viral protein (green), DAPI staining was used to detect DNA (blue) and phalloidin staining to detect actin filaments (red). Badger #1 (B5), badger #2 (B6). All the figures present the greyscale/inverted contrast at the left and the original view at the right
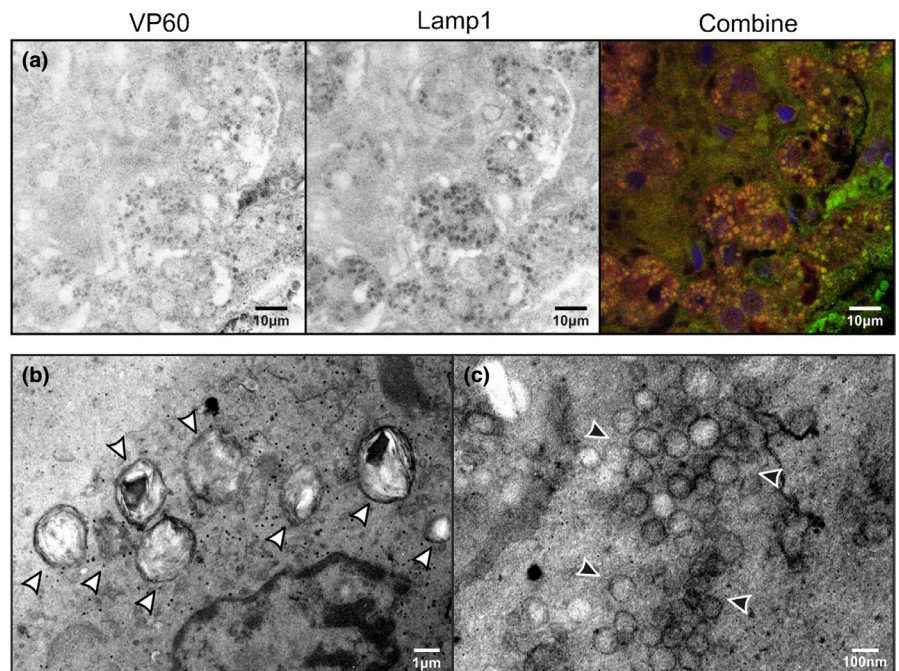
FIGURE 14 Immunolabelling of viral protein VP60 (a-left, greyscale), LAMP 1 (a-middle, greyscale) and combined (a-right). (b) Transmission electron microscopy showing clusters of lysosomal like structures (white arrowheads) within hepatocytes of badger (B5). (c) Vesicles (black arrowheads) were also observed in the hepatocytes of badger B5

TABLE 2 RT-qPCR amplification results and respective estimated viral loads per mg of tissue

	Viral copies/mg tissue[a]									
Organs	B1	B2	B3	B4	B5	B6	B7	B8	B9	B10
Liver	1.65×10^4	ND	ND	ND	2.99×10^6	1.14×10^4	1.69×105	2.84×10^4	2.09×10^5	6.02×10^5
Spleen	3.68×10^3	ND	ND	ND	4.25×10^5	1.66×10^6	5.53×10^5	3.34×10^4	5.01×10^5	1.92×10^5
Heart	7.13×10^4	ND	ND	ND	1.56×10^3	1.18×10^5	3.16×10^3	ND	8.14×10^2	4.57×10^3
Lungs	1.57×10^6	ND	ND	ND	1.21×10^6	5.15×10^3	5.78×10^2	1.19×10^3	1.67×10^3	ND
Duodenum	9.78×10^4	ND	ND	ND	3.31×10^3	4.75×10^5	7.47×10^4	5.50×10^3	8.67×10^3	1.75×10^4
Kidney	ND	ND	ND	ND	3.07×10^4	5.15×10^3	7.57×10^4	ND	5.63×10^2	8.90×10^3
Faeces	ND	ND	ND	ND	1.10×10^3	1.57×10^5	ND	ND	3.66×10^3	1.74×10^4
Mesenteric lymph node	NT	NT	NT	N	NT	NT	3.44×10^4	3.34×10^4	NT	NT

[a]According to the quantitative method described by Duarte et al. (2015). NT, not tested; ND, not detected; Darker cells correspond to higher viral loads.
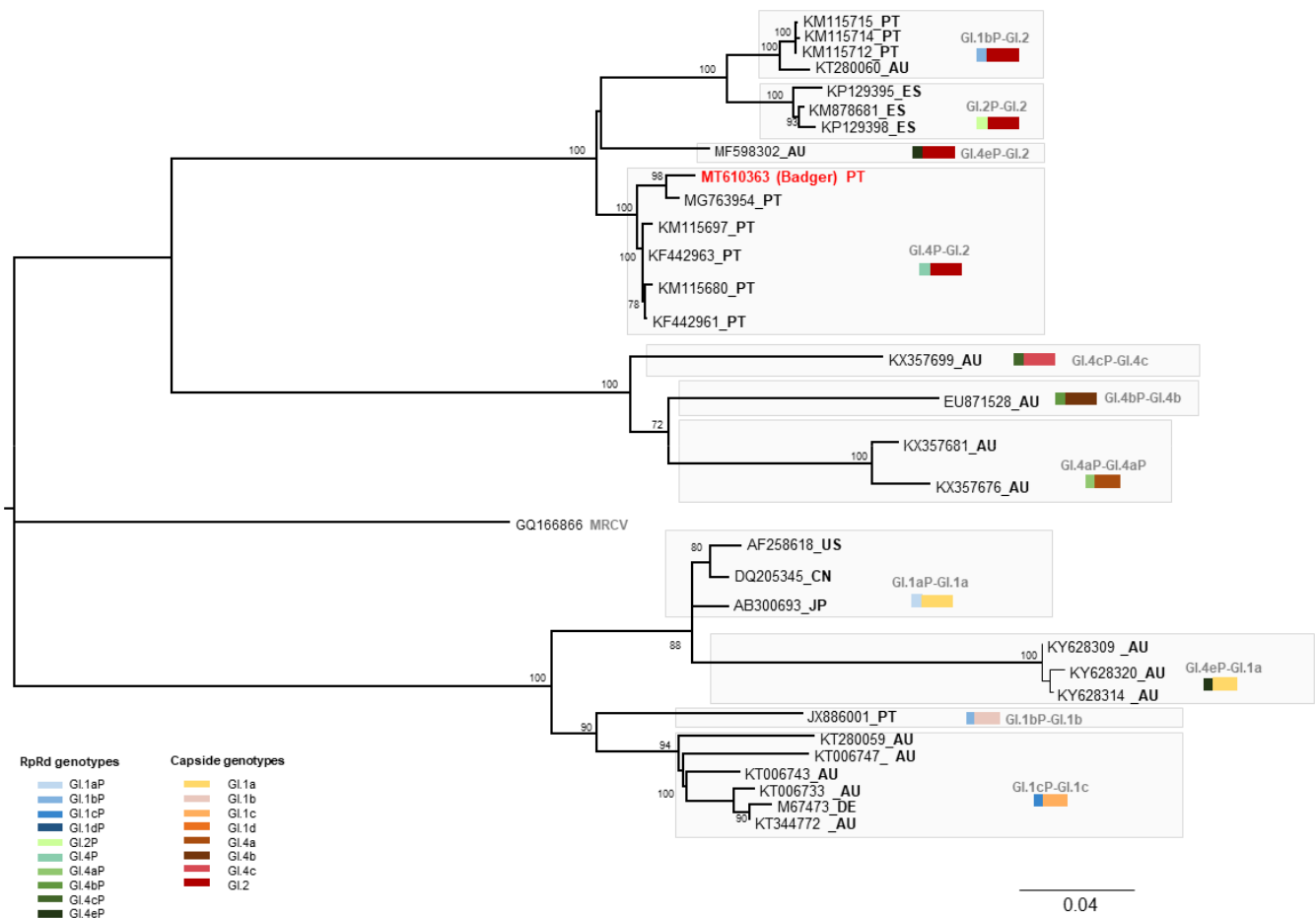
FIGURE 15 Unrooted phylogenetic tree inferred by using the maximum likelihood method and general time reversible model (Nei & Kumar, 2000). The tree with the highest log likelihood (−13922.65) is shown. A discrete gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 1.4546)). The rate variation model allowed for some sites to be evolutionarily invariable ([+I], 50.56% sites). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 32 nucleotide sequences. Codon positions included were 1st + 2nd+3rd + non-coding. There were a total of 2,191 positions in the final dataset. The analyses were conducted in MEGA X (Kumar et al., 2018), and the tree was edited with FigTree software V1.4.4

30 measures, Figure 16) compatible with RHDV particles whose diameter range from 27 to 40 nm (Capucci et al., 1991; Ohlinger et al., 1990).

The virions were detected in the nucleus and cytoplasm of hepatocytes, in the hepatic interstitium, in the alveolar ephitelial cells, in the heart myocytes, and in the cytoplasm of liver Kupfer cells. No
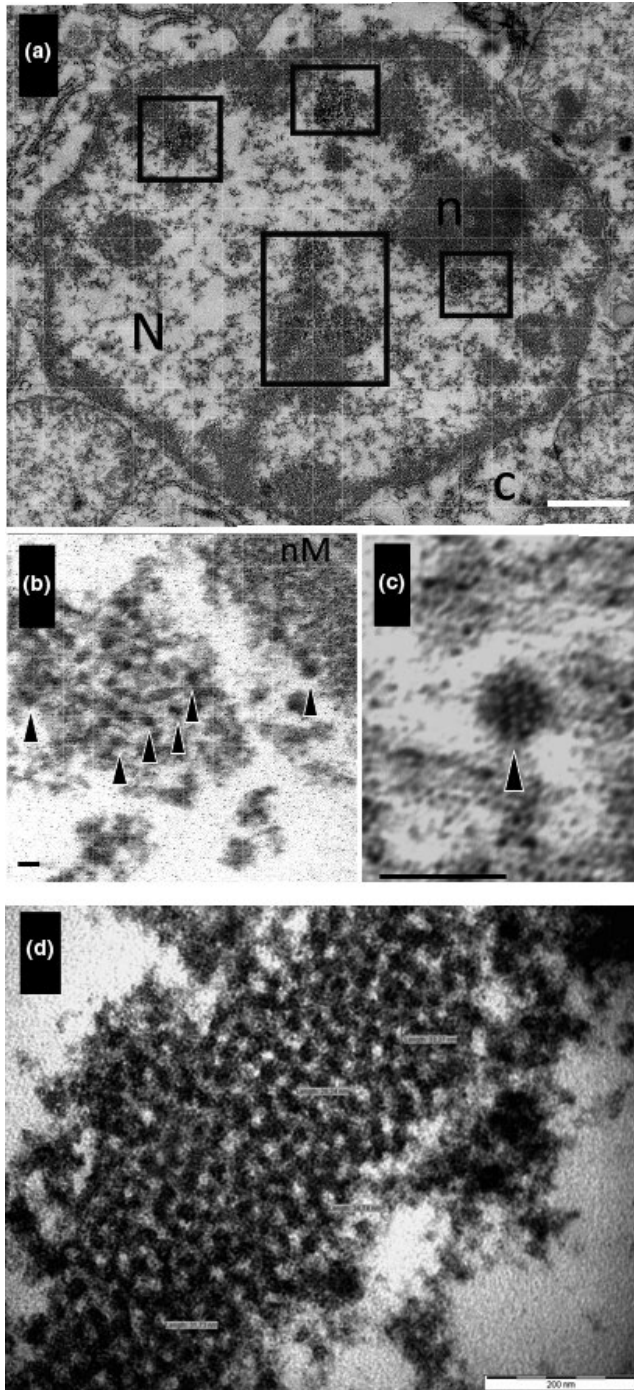
FIGURE 16 Transmission electron micrographs of badger B5 liver. (a) Low magnification showing the nucleus (N) of an hepatocyte, with an eccentric nucleolus (n), with several clusters of viral particles (insets). (b) High magnification of the nucleus showing clusters of particles (arrow heads), which differ from neighbouring chromatin (c) adjacent to nuclear membrane (nM). (c) Detail of a viral particle (arrowhead) suggesting an octagonal shape. Bars: 1 μm (white bar), 50 nm (black bar). (d) High magnification of an apparent intranuclear viral factory, with viral particles measuring from 26 to 38 nm, compatible with caliciviruses. Bar: 200 nm

virion-like structures were observed in a badger (unnumbered, used as negative control), that tested

RHDV2-negative on the RT-qPCR.

3.8 | Virological analyses besides RHDV2

None of badgers B1, B5 to B10 was positive for all the other viruses tested .

3.9 | Bacteriological analysis

Low numbers of *Micrococcus* sp. and *Staphylococcus sciuri* were isolated from the liver and lung of badger B5. The remaining organs showed no aerobic or anaerobic bacterial growth.

Regarding badger B6, *Escherichia coli* was isolated from the liver and spleen, and low numbers of *Clostridium* sp. were isolated from the liver, spleen and kidney.

In the samples collected from badgers B7 and B8, low quantities of *Klebsiella oxytoca* and *Klebsiella pneumonia* ssp. *pneumoniae* were isolated, respectively. No *Mycobacterium* spp. was detected in the badgers.

3.10 | Parasitological analysis

No ecto- or endoparasites were found in badgers B1, B7 and B8. In Badger B6, fleas were identified as *Pulex irritans*. For badgers B5 and B6, hookworm eggs were found through the flotation method. In the later, adult forms of hookworms (family Ancylostomatidae) were also detected during the analysis of the gastrointestinal content. *Isospora* spp., *Uncinaria criniformis* and nematodes from Capillariidae family were identified in badger B6, and *Capillaria* sp. in badger B10.

4 | DISCUSSION

Our work was conducted in the scope of a national surveillance programme for RHDV2 in wild rabbits and sympatric species (Action Plan for the Control of Rabbit Haemorrhagic Disease Virus, Dispatch 4757/17 of 30st May MAFDR). As sympatric species, European badgers were also included in the monitoring programme, which is why those found dead were sampled for investigation. However, the poor quality of these opportunistic biological samples poses major challenges to the laboratory diagnosis, overcome through the establishment of a comprehensive diagnosis strategy combining several molecular and antigen detection methods, sequencing analysis and electron microscopy observation. The serological investigation was not possible due to the lack of fresh blood samples.

The methodological strategy adopted for this study was as follows: tissue samples were firstly tested by RT-qPCR (Duarte et al., 2015) allowing the subsequent selection of the RHDV2 positive badgers for further analyses. Of these, only the badgers showing milder autolysis were elected for RHDV2 sequencing analysis, IHC and TEM. The exception was made for badger B1, the only material available for the first two years of this investigation, and for which, tissues were used for testing, despite severe autolysis.

To exclude the possibility of the microscopic lesions being a consequence of other infectious agents rather than RHDV2, samples were tested for additional pathogens. Several potential Eurasian badger viruses (canine distemper virus, canine and feline parvovirus, canine, feline and porcine coronaviruses, type A rotavirus, Ausjesky disease virus and other herpesviruses) were investigated by molecular methods in the appropriate tissues. All samples tested negative. Although Eurasian badgers can be susceptible to several pathogenic bacteria (Franzo et al., 2017; Judge et al., 2017; Sin et al., 2014; Wragg et al., 2011), the isolates identified in this study probably represent contaminants, as histopathological analysis did not show any bacteria in the tissues or tissue alterations that could be related with the bacterial species isolated. *Micrococcus* and *Staphylococcus* identified in badger B5 are common skin commensals of animals and humans, while *Clostridium* and *Escherichia coli*, identified in badger B6, belong to their intestinal microbiota (Quinn et al., 2011)). Also, to the best of our knowledge, the first three genera have not yet been related to bacterial infections in *Meles meles*, while *E. coli* was reported in badgers' bronchoalveolar lavages (McCarthy et al., 2009).

The RHDV2 viral loads found in the tested organs (Table 2) ranged from 1.19×10^3 to 2.99×10^6, despite kidney, heart and lungs tested negative in some badgers. These values were lower than the ones described for domestic and wild rabbit (Carvalho et al., 2017), but are compatible with a systemic infection, following the possible ingestion of RHDV2-positive rabbits. However, the mere passage of the virus through the gastrointestinal tract, without surpassing this barrier, would result in faeces and intestine and, at most, the mesenteric lymph nodes testing positive to RHDV2, while all the other organs would test negative. That was not the case since liver, lungs and spleen showed consistent and relatively high viral loads in several badgers.

Amplification and sequencing analysis of a 2,188-nt long fragment comprising part of the RdRp gene and the complete the VP60 gene, confirmed the presence of RHDV2-RNA in the tissues of badgers B5 and B6. Despite only partial sequences of the RdRp gene were obtained for badgers B1, B7, B8 and B9 (as summarized in Table S1), they also confirmed the RT-qPCR results, demonstrating unequivocally the presence of viral RNA in the badgers' tissues.

The molecular data was supported by the immunohistochemistry, immunofluorescence and dot blot results, alongside the gross pathology and histopathological lesions. Dot blot analysis of liver homogenates from badger B5 and B6 confirmed the presence of RHDV or RHDV2 protein in these organs. Curiously, the dot blot signal in B6 was stronger than in B5, despite the RT-qPCR results showed higher viral loads (deduced from the number of RNA copies) in B5 tissues. However, dot blot is not a quantitative technique and tissue homogenizations for dot blot were not carried out as accurately as for RNA extraction.

Gross pathology and histopathology suggest that multiorgan failure (heart, kidney and lung) could account for the proximal cause of death. The presence of iron deposits in the spleen, liver and lungs indicate haemorrhagic events, possibly due to chronic blood losses, more severe in the case of badgers B5 and B8, whose spleens were highly infiltrated with haemosiderin–laden macrophages. Although

liver necrosis in badgers was not as evident as normally seen in rabbits infected with RHDV2 (Abade dos Santos et al., 2017; Soliman et al., 2016; Umer et al., 2017), hepatocytes showed hydropic degeneration and individual cell necrosis. Another difference from rabbits is the lymphoid hyperplasia that was observed in three of four analysed badgers. This may indicate a more adjusted response to the viral infection, in accordance with the lower viral loads observed in the badgers, compared with rabbits.

Altogether, despite not so severe, the lesions observed may be accounted for a poor clinical evolution, leading to a moribund status incompatible with survival in the wild, taking into account that a weakened animal hardly survives, especially if it need to prey for food.

Additionally, the lower severity of the lesions compared to rabbits seems to indicate a less aggressive viral infection in this species. Despite different GI.2 strains showing different virulence were described, there is no data relating possible differences in the severity and type of lesions induced in rabbits with different strains. The pattern and severity of lesions may also change according to the clinical evolution, which in turn could vary due to intrinsic individual or species-related resistance and natural or acquired immunity to the infection. Nonetheless, if the course of the infection is less fulminating in badgers than in rabbits, probably because badger is not a usual host of this virus, it may favour the dissemination of RHDV2 in the wild as a disease with a longer course will allow virus excretion in the faeces for longer periods.

Finally, by transmission electron microscopy we demonstrated the presence of viral particles compatible with RHDV2 in several badgers' tissues. Despite the poor preservation condition of the tissues, viral factories were observed providing evidence of active replication of the virus in the cells. Furthermore, the observation of viral factories in the hepatocyte nucleus revealed, we believe that for the first time, the RHDV2 passage through the nucleus during replication. This observation had been previously reported in RHDV-infected hepatocytes by Marcato et al. (1991). The meaning of this finding is still unclear, given that a nuclear phase was not described in the replicative cycle of Caliciviridae, despite the presence of viral factories in the nucleus of infected cells was previously described in feline calicivirus infection (Pesavento et al., 2004). Regarding the present study, although the breakdown of the cell nuclear membrane may have allowed virions to pass into the nucleus as suggested before (Pesavento et al., 2004), since virions were exclusively grouped resembling viral factories, this scenario is unlikely. On the other hand, these clusters of virions within the nucleus were observed in several cells, where virions were not present in the cytoplasm . The presence of virions compatible with RHDV2 has already been observed by our team in the nucleus of hepatocyte cells from rabbits that died infected with RHDV2 (data not shown).

By immunofluorescence, we confirmed the presence of many lysosomes or autophagosome structures in the hepatocyte cytoplasm, containing mature virions (Figure 15). As previously described (Vallejo et al., 2014), after RHDV infection, the host cells initiate a rapid autophagic response that than declines, culminating in apoptosis. The vesicles shown in Figure 15 were also observed in RHDV-infected liver cells by these same authors.

During the +Coelho project, that operacionalises the Action Plan, a total of 25 dead rabbits from the Santarém district were sampled between March 2017 and March 2020, of which 15 (60%) were positive for RHDV2.

To the best of our knowledge, no Lagoviruses had ever been described in wild badgers, in natural or experimental infections. In this study, we provide evidence that RHDV2 may have crossed another species barrier from the Leporidae family (order Lagomorpha) to the Mustelidae family (order Carnivora), a remarkable paradigm shift for the pathogenic potential of this virus.

More studies are now needed to understand whether these cases represent a set of individual spillover events or a true species jump. The detection of RHDV2 in badgers along three consecutive years (2017–2020) supports, in our opinion, the second scenario, but further investigations are necessary to confirm the geographic and temporal extension of this event.

Another future interesting study concerns the research of other calicivirus in this species, namely viruses close to non-pathogenic Lagoviruses, which may serve as donors of genetic material and thus contribute to the evolution of *Lagovirus europaeus* and the emergence of new pathogenic strains.

Experimental infections with European badgers would help to clarify the baseline level of susceptibility to infection; however, they are extremely complicated in protected species. Nonetheless, priority investigations should include further RHDV2 monitoring in badgers in the Santarém District as well as in other geographical areas, to clarify viral evolution within this host species, to evaluate the pathogenic impact of RHDV2 strains adapted to badgers towards rabbits and to understand the role of the Eurasian badger as a reservoir host for RHDV2.

CONFLICT OF INTEREST

The authors declare no competing interests.

AUTHORS' CONTRIBUTIONS

Abade dos Santos, F.A. contributed to conceptualization, investigation, methodology, resources and writing – original draft preparation. Pinto, A. contributed to formal analysis, investigation, methodology and resources. Burgoyne, T. contributed to investigation and resources. Dalton, K.P. contributed to investigation, resources and validation. Carvalho C.L. contributed to investigation. Ramilo, D. contributed to investigation. Carneiro, C. contributed to investigation. Carvalho, T. contributed to investigation, and writing – review and editing. Peleteiro, M.C. contributed to funding acquisition, supervision, visualization, validation, writing – review and editing. Parra, F. contributed to conceptualization, funding acquisition, supervision, validation, writing – review and editing. Duarte, M.D. contributed to conceptualization, funding acquisition, investigation, project administration, supervision, validation and writing – review and editing.

ETHICAL STATEMENT

This study did not use live animals and was carried out within the scope of a National Action Plan (Projects +Coelho1 and +Coelho 2, Dispatch no. 4757/2017 of 31 May), with the legal authorizations from the National Authority—the Institute for Nature Conservation and Forests (Instituto da Conservação da Natureza e das Florestas, I.P., ICNF).

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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REFERENCES

Abade dos Santos, F. A., Carvalho, C., Nuno, O., Correia, J. J., Henriques, M., Peleteiro, M. C., Fevereiro, M., & Duarte, M. D. (2017). Detection of rabbit Haemorrhagic disease virus 2 during the wild rabbit (*Oryctolagus cuniculus*) eradication from the Berlengas archipelago, Portugal. *BMC Veterinary Research*, *13*, 336. https://doi.org/10.1186/s12917-017-1257-3

Abrantes, J., Droillard, C., Lopes, A. M., Lemaitre, E., Lucas, P., Blanchard, Y., Marchandeau, S., Esteves, P. J., & Le Gall-Reculé, G. (2020). Recombination at the emergence of the pathogenic rabbit haemorrhagic disease virus Lagovirus europaeus/GI.2. *Scientific Reports*, *10*, 1–12. https://doi.org/10.1038/s41598-020-71303-4

Downie T. (1990). Theory and Practice of Histological Techniques Edited by J.D. Bancroft & A. Stevens, Churchill Livingstone, Edinburgh, 740 pages, £55.00. *Histopathology*, *17*, (4), 1–740. http://dx.doi.org/10.1111/j.1365-2559.1990.tb00755.x.

Calvete, C., Mendoza, M., Sarto, M., Jiménez de Bagués, M. P., Luján, L., Molín, J., Calvo, A. J., Monroy, F., & Calvo, J. H. (2019). Detection

of rabbit hemorrhagic disease virus GI. 2 / RHDV2 / B in the Mediterranean pine vole (*Microtus Duodecimcostatus*) and white-toothed shrew (*Crocidura russula*). *Journal of Wildlife Diseases*, 55, 1–6. https://doi.org/10.7589/2018-05-124

Camarda, A., Pugliese, N., Cavadini, P., Circella, E., Capucci, L., Caroli, A., Legretto, M., Mallia, E., & Lavazza, A. (2014). Detection of the new emerging rabbit haemorrhagic disease type 2 virus (RHDV2) in Sicily from rabbit (*Oryctolagus cuniculus*) and Italian hare (*Lepus corsicanus*). *Research in Veterinary Science*, 97, 642–645. https://doi.org/10.1016/j.rvsc.2014.10.008

Capucci, L., Fusi, P., Lavazza, A., Pacciarini, M. L., & Rossi, C. (1996). Detection and preliminary characterization of a new rabbit cali-civirus related to rabbit hemorrhagic disease virus but nonpatho-genic. *Journal of Virology*, 70, 8614–8623. https://doi.org/10.1128/JVI.70.12.8614-8623.1996

Capucci, L., Scicluna, M., & Lavazza, A. (1991). Diagnosis of viral hae-morrhagic disease of rabbits and the European brown hare syn-drome. *Revue Scientifique et Technique*, 10, 347–370. https://doi.org/10.20506/rst.10.2.561

Carvalho, C. L., Duarte, E. L., Monteiro, M., Botelho, A., Albuquerque, T., Fevereiro, M., Henriques, A. M., Barros, S. S., & Duarte, M. D. (2017). Challenges in the rabbit haemorrhagic disease 2 (RHDV2) molecular diagnosis of vaccinated rabbits. *Veterinary Microbiology*, 198, 43–50. https://doi.org/10.1016/j.vetmic.2016.12.006

Chiari, M., Molinari, S., Cavadini, P., Bertasi, B., Zanoni, M., Capucci, L., & Lavazza, A. (2016). Red foxes (*Vulpes vulpes*) feeding brown hares (*Lepus europaeus*) infected by European brown hare syndrome virus (EBHSv) might be involved in the spread of the virus. *European Journal of Wildlife Research*, 62(6), 761–765. https://doi.org/10.1007/s10344-016-1055-4

Dalton, K. P., Arnal, J. L., Benito, A. A., Chacón, G., Martín Alonso, J. M., & Parra, F. (2018). Conventional and real time RT-PCR assays for the detection and differentiation of variant rabbit hemorrhagic disease virus (RHDVb) and its recombinants. *Journal of Virological Methods*, 251, 118–122. https://doi.org/10.1016/j.jviromet.2017.10.009

Di Profio, F., Melegari, I., Sarchese, V., Robetto, S., Bermudez Sanchez, S., Carella, E., Orusa, R., Cavadini, P., Lavazza, A., Marsilio, F., Martella, V., & Di Martino, B. (2017). Potential role of wolf (*Canis lupus*) as passive carrier of European brown hare syndrome virus (EBHSV) Research in Veterinary Science Potential role of wolf (*Canis lupus*) as passive carrier of European brown hare syndrome virus (EBHSV). *Research in Veterinary Science*, 117, 81–84. https://doi.org/10.1016/j.rvsc.2017.11.016

Duarte, M. D., Carvalho, C. L., Barros, S. C., Henriques, A. M., Ramos, F., Fagulha, T., Luís, T., Duarte, E. L., & Fevereiro, M. (2015). A real time Taqman RT-PCR for the detection of rabbit hemorrhagic disease virus 2 (RHDV2). *Journal of Virological Methods*, 219, 90–95. https://doi.org/10.1016/j.jviromet.2015.03.017

Fedriani, J. M., Palomares, F., & Delibes, M. (1999). Niche relations among three sympatric Mediterranean carnivores. *Oecologia*, 121, 138–148. https://doi.org/10.1007/s004420050915

Franzo, G., Massi, P., Tucciarone, C. M., Barbieri, I., Tosi, G., Fiorentini, L., Ciccozzi, M., Lavazza, A., Cecchinato, M., & Moreno, A. (2017). Think globally, act locally: Phylodynamic reconstruction of infectious bron-chitis virus (IBV) QX genotype (GI-19 lineage) reveals different popu-lation dynamics and spreading patterns when evaluated on different epidemiological scales. *PLoS One*, 12, 1–20. https://doi.org/10.1371/journal.pone.0184401

González, A. M. (2019). Caracterización antigénica y diseño de vacunas frente a la nueva variante del virus de la enfermedad hemorrágica del conejo. PhD thesis. University of Oviedo.

Judge, J., Wilson, G. J., Macarthur, R., McDonald, R. A., & Delahay, R. J. (2017). Abundance of badgers (*Meles meles*) in England and Wales. *Scientific Reports*, 7, 1–8. https://doi.org/10.1038/s41598-017-00378-3

Kumar, S., Stecher, G., Li, M., Knyaz, C., & Tamura, K. (2018). Molecular evolutionary genetics analysis across computing platforms. *Molecular Biology and Evolution*, 35(6), 1547–1549.

Le Gall-Recule, G., Zwingelstein, F., Boucher, S., Le Normand, B., Plassiart, G., Portejoie, Y., Decors, A., Bertagnoli, S., Guerin, J.-L., & Marchandeau, S. (2011). Detection of a new variant of rabbit haem-orrhagic disease virus in France. *The Veterinary Record*, 168, 137–138. https://doi.org/10.1136/vr.d697

Le Gall-Reculé, G., Zwingelstein, F., Fages, M. P., Bertagnoli, S., Gelfi, J., Aubineau, J., Roobrouck, A., Botti, G., Lavazza, A., & Marchandeau, S. (2011). Characterisation of a non-pathogenic and non-protective infectious rabbit lagovirus related to RHDV. *Virology*, 410, 395–402. https://doi.org/10.1016/j.virol.2010.12.001

Le Pendu, J., Abrantes, J., Bertagnoli, S., Guitton, J.-S., Le Gall-Reculé, G., Lopes, A. M., Marchandeau, S., Alda, F., Almeida, T., Célio, A. P., Bárcena, J., Burmakina, G., Blanco, E., Calvete, C., Cavadini, P., Cooke, B., Dalton, K., Delibes Mateos, M., Deptula, W., … Esteves, P. (2017). Proposal for a unified classification system and nomenclature of lagoviruses. *Journal of General Virology*, 98, 1658–1666. https://doi.org/10.1099/jgv.0.000840

Liu, S., Xue, H., Pu, B., & Qian, N. (1984). A new viral disease in rabbit. *Animal Husbandry & Veterinary Medicine*, 16, 253–255.

Lopes, A. M., Marques, S., Silva, E., Magalhães, M. J., Pinheiro, A., Alves, P. C., Le Pendu, J., Esteves, P. J., Thompson, G., & Abrantes, J. (2014). Detection of RHDV strains in the Iberian hare (*Lepus granatensis*): Earliest evidence of rabbit lagovirus cross-species in-fection. *Veterinary Research*, 45, 1–7. https://doi.org/10.1186/s13567-014-0094-7

Mahar, J. E., Hall, R. N., Peacock, D., Kovaliski, J., Piper, M., Mourant, R., & Huang, N. (2018). Rabbit hemorrhagic disease virus 2 (RHDV2; GI.2) is replacing endemic strains of RHDV in the Australian landscape within 18 months of its arrival. *Journal of Virology*, 2, 1–15. https://doi.org/10.1128/JVI.01374-17

Marcato, P., Benazzi, C., Vecchi, G., Galeotti, M., Della Salda, L., Sarli, G., & Lucidi, P. (1991). Clinical and pathological features of viral haem-orrhagic disease of rabbits and the European brown hare syndrome. *Revue Scientifique et Technique*, 10, 371–392.

McCarthy, G., Shiel, R., O'Rourke, L., Murphy, D., Corner, L., Costello, E., & Gormley, E. (2009). Bronchoalveolar lavage cytology from cap-tive badgers. *Veterinary Clinical Pathology*, 38, 381–387. https://doi.org/10.1111/j.1939-165X.2009.00127.x

Meyers, G., Wirblich, C., & Thiel, H. J. (1991). Rabbit hemorrhagic disease virus-molecular cloning and nucleotide sequencing of a calicivirus genome. *Virology*, 184, 664–676. https://doi.org/10.1016/0042-6822(91)90436-F

Mutze, G., Bird, P., Kovaliski, J., Peacock, D., Jennings, S., & Cooke, B. (2002). Emerging epidemiological patterns in rabbit haemorrhagic disease, its interaction with myxomatosis, and their effects on rab-bit populations in South Australia. *Wildlife Research*, 29, 577–590. https://doi.org/10.1071/WR00100

Nei, M., & Kumar, S. (2000). *Molecular evolution and phylogenetics*. New York: Oxford University Press.

Neimanis, A. S., Ahola, H., Pettersson, U. L., Lopes, A. M., Abrantes, J., Zohari, S., Esteves, P. J., & Gavier-widén, D. (2018). Overcoming spe-cies barriers: An outbreak of Lagovirus europaeus GI. 2 / RHDV2 in an isolated population of mountain hares (*Lepus timidus*), *BMC Veterinary Research*, 1–12.

Ohlinger, V. F., Haas, B., Meyers, G., Weiland, F., & Thiel, H.-J. (1990). Identification and characterization of the virus causing rabbit hem-orrhagic disease. *Journal of Virology*, 64, 3331–3336.

OIE. (2019). *Rabbit haemorrhagic disease (Technical disease card)* (pp. 1–7). World Organisation for Animal Health https://www.oie.int/filea dmin/Home/eng/Animal_Health_in_the_World/docs/pdf/Disea se_cards/RHD.pdf.

Peleteiro, M. C., Ferreira da Silva, J., Dias-Pereira, P., Carvalho, T., Faustino, A., Correia, J., Pissarra, H., & Stilwell, G. (2016). *Manual de Necrópsia Veterinária1a*. (Lidel, Ed.). Lisboa.

Pesavento, P. A., Maclachlan, N. J., Dillard-Telm, L., Grant, C. K., & Hurley, K. F. (2004). Pathologic, immunohistochemical, and electron microscopic findings in naturally occurring virulent systemic feline calicivirus infection in cats. *Veterinary Pathology*, *41*, 257–263. https://doi.org/10.1354/vp.41-3-257

Puggioni, G., Cavadini, P., Maestrale, C., Scivoli, R., Botti, G., Ligios, C., Le Gall-Reculé, G., Lavazza, A., & Capucci, L. (2013). The new French 2010 Rabbit Hemorrhagic Disease Virus causes an RHD-like disease in the Sardinian Cape hare (Lepus capensis mediterraneus). *Veterinary Research*, *44*, 1–7. https://doi.org/10.1186/1297-9716-44-96

Quinn, P., Markey, B., Leonard, F., Hartigan, P., Fanning, S., & Fitzpatrick, E. (2011). Animal diversity, natural history and conservation – Volume 22nd Edition. In L. M. Rosalino, T. Sales-Luis, M. Pinto Basto, N. M. Pedroso, L. Tavares, C. L. Vilela, & M. Oliveira (Eds.), *Veterinary microbiology and microbial disease* (pp. 1–17). Wiley-Blackwell.

Silvério, D., Lopes, A. M., Melo-ferreira, J., Magalhaes, M. J., Ponterroso, P., Serronha, A., Maio, E., Alves, P. C., Esteves, P. J., & Abrantes, J. (2018). Insights into the evolution of the new variant rabbit haemorrhagic disease virus (GI. 2) and the identification of novel recombinant strains. *Transboundary and Emerging*, *00*, 1–10. https://doi.org/10.1111/tbed.12830

Sin, Y. W., Annavi, G., Dugdale, H. L., Newman, C., Burke, T., & MacDonald, D. W. (2014). Pathogen burden, co-infection and major histocompatibility complex variability in the European badger (*Meles meles*). *Molecular Ecology*, *23*, 5072–5088. https://doi.org/10.1111/mec.12917

Soliman, M., Rhaman, M., Samy, M., Mehana, O., & Nasef, S. (2016). Molecular, clinical and pathological studies on viral rabbit hemorrhagic disease. *Alexandria Journal of Veterinary Science.*, *48*, 20. https://doi.org/10.5455/ajvs.207505

Strive, T., Wright, J. D., & Robinson, A. J. (2009). Identification and partial characterisation of a new lagovirus in Australian wild rabbits. *Virology*, *384*, 97–105. https://doi.org/10.1016/j.virol.2008.11.004

Umer, F., Riasat, W. U., Asma, L., Aamer, B. Z., Javid, I. D., & Hamid, I. (2017). Gross pathological findings of rabbit hemorrhagic disease (RHD) in two (02) cases case report gross pathological findings of rabbit hemorrhagic disease (RHD) in two. *Research Journal for Veterinary Practitioners*, *1*(4), 39–40.

USDA. (2020). Emerging risk notice. Rabbit Hemorrhagic Disease Virus, Type 2.

Vallejo, D., Crespo, I., San-Miguel, B., Álvarez, M., Prieto, J., Tuñón, M. J., & González-Gallego, J. (2014). Autophagic response in the Rabbit Hemorrhagic Disease, an animal model of virally-induced fulminant hepatic failure. *Veterinary Research*, *45*, 1–13. https://doi.org/10.1186/1297-9716-45-15

Velarde, R., Cavadini, P., Neimanis, A., Cabezón, O., Chiari, M., Gaffuri, A., Lavín, S., Grilli, G., Gavier-Widén, D., Lavazza, A., & Capucci, L. (2017). Spillover events of infection of brown hares (*Lepus europaeus*) with rabbit haemorrhagic disease type 2 virus (RHDV2) caused sporadic cases of an European brown hare syndrome-like disease in Italy and Spain. *Transboundary and Emerging Disease*, *64*, 1750–1761. https://doi.org/10.1111/tbed.12562

Wragg, P. N., Strugnell, B. W., Whatmore, A. M., & Foster, G. (2011). Arcanobacterium haemolyticum in a badger (*Meles meles*). *Journal of Veterinary Diagnostic Investigation*, *23*, 1234–1235. https://doi.org/10.1177/1040638711425585

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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Are recombinant RHDV2 strains responsible for infections in clinical practice (rabbit pets) and are the available vaccines protective?

Scientific publication 7

A Potential Atypical Case of Rabbit Haemorrhagic Disease in a Dwarf Rabbit

■■■

Until the end of 2019, the only vaccine available for pet rabbits in Portugal was not protective for RHDV2. In this work we report a case of an atypical clinical picture in an animal vaccinated after infection with a recombinant strain of RHDV2.

animals

MDPI

Brief Report

A Potential Atypical Case of Rabbit Haemorrhagic Disease in a Dwarf Rabbit

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Simple Summary: We report an unusual clinical case in a pet rabbit vaccinated against rabbit haemorrhagic disease virus (RHDV, GI.1), that developed a prolonged hepatic disease, and was diagnosed RHDV2 (GI.2) positive post-mortem. This finding is a warning to all veterinarians that rabbit haemorrhagic disease should also be considered for differential diagnosis despite the history of RHDV vaccination and the need to update vaccination programs against the current RHDV2 circulating strains.

check for **updates**

Abstract: Rabbit haemorrhagic disease (RHD) is a highly contagious infectious disease of European wild and domestic rabbits. Rabbit haemorrhagic disease virus (RHDV, GI.1) emerged in 1986 in Europe, rapidly spreading all over the world. Several genotypes of RHDV have been recognised over time, but in 2010, a new virus (RHDV2/RHDVb, GI.2) emerged and progressively replaced the previous RHDV strains, due to the lack of cross-immunity conferred between RHDV and RHDV2. RHDV2 has a high mutation rate, similarly to the other calivirus and recombines with strains of RHDV and non-pathogenic calicivirus (GI.4), ensuring the continuous emergence of new field strains. Although this poses a threat to the already endangered European rabbit species, the available vaccines against RHDV2 and the compliance of biosafety measures seem to be controlling the infection in the rabbit industry Pet rabbits, especially when kept indoor, are considered at lower risk of infections, although RHDV2 and myxoma virus (MYXV) constitute a permanent threat due to transmission via insects. Vaccination against these viruses is therefore recommended every 6 months (myxomatosis) or annually (rabbit haemorrhagic disease). The combined immunization for myxomatosis and RHDV through a commercially available bivalent vaccine with RHDV antigen has been extensively used (Nobivac® Myxo-RHD, MSD, Kenilworth, NJ, USA). This vaccine however does not confer proper protection against the RHDV2, thus the need for a rabbit clinical vaccination protocol update. Here we report a clinical case of hepatitis and alteration of coagulation in a pet rabbit that had been vaccinated with the commercially available bivalent vaccine against RHDV and tested positive to RHDV2 after death. The animal developed a prolonged and atypical disease, compatible with RHD. The virus was identified to be an RHDV2 recombinant strain, with the structural backbone of RHDV2 (GI.2) and the non-structural genes of non-pathogenic-A1 strains (RCV-A1, GI.4). Although confirmation of the etiological agent was only made after death, the clinical signs and analytic data were very suggestive of RHD.

Keywords: European rabbit; *Oryctolagus cuniculus*; pet rabbit; rabbit haemorrhagic disease; atypical clinical course; subacute

1. Introduction

Originating in the Iberian Peninsula [1], the European rabbit was widely introduced first in Europe, and subsequently in all other continents except Antarctica. The European domestic rabbit has been used as an important meat and fur resource. Artificial selection of small breeds of this species (*Oryctolagus cuniculus*), weighing less than 2 kg, also generated an increasingly popular pet-rabbit trade. Pet rabbits can be affected by the same diseases found in the rabbit industry or wild rabbit populations. However, the indoor lifestyle decreases the risk of contact with common rabbit diseases such as myxomatosis and rabbit haemorrhagic disease (RHD).

RHD is a highly contagious infectious disease caused by a virus from the Caliciviridae family, genus *Lagovirus*, which targets hepatocytes and cells of the mononuclear phagocytic system (e.g., Kuppfer cells and alveolar macrophages) [2,3]. The disease was first identified in 1984 in China and quickly spread to other continents, evolving into different genotypes (GI.1a (RHDVa or G6), GI.1b (G1), GI.c (G2) and GI.1d (G3–G5)) [4,5]. Along with myxomatosis, this disease led to an abrupt decrease in the European rabbit wild populations [6].

In 2010, a distinct virus (both genetic and antigenically) related to RHDV emerged in France [7]. This new virus, referred to as RHDV2 or RHDVb, induced a disease very similar to that caused by RHDV strains and it became also known as *Lagovirus europaeus* GI.2 [5].

More recently, natural recombinant RHDV2 strains were identified containing the structural proteins of RHDV2 (VP60 and minor protein-encoding genes), but the non-structural proteins from either non-pathogenic calicivirus (NP-CV GI.4 and GI.3)). Furthermore, the non-structural proteins from the RHDV strains (GI.1b) were also found in RHDV2 recombinants [8]. Figure 1 represents the genomic organization of RHDV2 RNA.
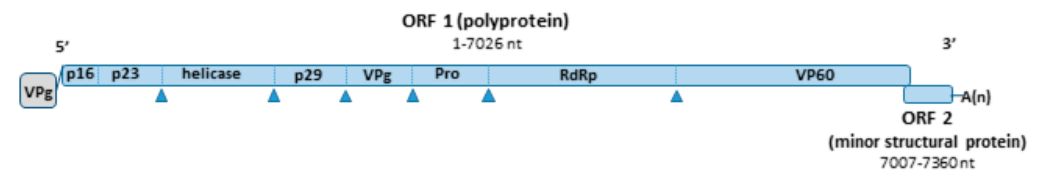
Figure 1. Simplified schematic representation of the genomic RNA of RHDV2. Nucleotide positions were calculated from sequence KF442964.2. The genome includes two ORFs. ORF1 encodes a polyprotein that is cleaved to form the non-structural proteins p16, p23, the helicase, p29, VPg, a protease and the viral polymerase, and the major structural protein VP60. ORF 2 encodes a minor structural protein VP10.

Different clinical presentations and disease progression can occur with RHDV2 [9–12] and those variations can be associated with strain. The incubation period of RHD ranges between 1 and 3 days. In peracute forms, sudden death may occur within 12 hours after infection. In acute and subacute forms of RHD, different clinical signs can be observed, such as anorexia, mucosal congestion, neurologic signs, cyanosis, dyspnoea, foamy haemorrhagic epistaxis, ocular haemorrhage and others [13]. In chronically infected rabbits, the onset of anorexia, lethargy and jaundice usually precedes death in about 1 to 3 weeks [13]. Chronic or subclinical courses are more frequent in rabbits infected by RHDV2 than by RHDV.

Haemorrhagic diathesis is found during the disease, with disseminated intravascular coagulation (DIC), associated with alterations in blood coagulation and terminating in multi-organ failure and death [14]. The pathogenesis of DIC in RHD is still unknown. The DIC is assumed to occur by a distortion of both external and internal blood coagulation activation paths, leading to extended One-Stage Prothrombin Time (OSPT) and Activated Partial Thromboplastin Time (APTT) [15–17]. During the disease, a thrombocytopenia and reduced platelet aggregation is observed, alteration of the activity of coagulation factors

V, VII and X, as well as increased volume of soluble fibrin and its degradation products (D-dimers) [15–17].

During an RHDV2 outbreak, more than 10% of the infected rabbits may show a chronic or subclinical evolution of the disease, with severe and generalised jaundice, loss of weight, and lethargy. These animals often die some weeks later, due to liver disease [18]. Although the study of the disease has been expanded by animal experimentation, the characterisation of natural infection is far less known [10,11,19,20].

The terms peracute, acute and chronic are of clinical use but not always reflected in distinct necropsy findings and specific histopathological patterns, meaning that it is possible to find sudden death associated with peracute widespread hepatocellular necrosis or with more chronic changes such as inflammation or fibroplasia [10].

Veterinarians should be aware that despite necropsy and histopathology findings may be suggestive of RHD, since microscopic changes are usually present and, in many cases, macroscopic changes can also be seen [10], laboratory confirmation is always required for a conclusive diagnosis.

Laboratory testing can be done by molecular methods (e.g., RT-PCR), ELISA or electron microscopy. However, the rapid and debilitating evolution of the disease, together with the shortage of direct access to these techniques by clinicians, poor reporting of RHD in pet rabbits and possible lack of awareness among clinicians, contributes to the almost non-existent knowledge of the prevalence of RHD in pets in Portugal.

After the emergence of RHDV2 in 2010, this virus rapidly replaced the previous strains that soon were no longer reported in Europe. The humoral immunity acquired by natural- or vaccine-induced RHDV strains do not confer proper cross-protection against RHDV2 strains [21,22]. Until 2016, when ERAVAC, Hipra, Girona, Spain was commercialised, all vaccines available against RHDV2 were directed to the industrial market (sold in multi-dose bottles), and therefore not adequate for vaccination of pet rabbits. In November 2019, a new single-dose bottle vaccine against myxoma virus, RHDV and RHDV2 was also made available in Europe (Nobivac® Myxo-RHD Plus, MSD, Kenilworth, NJ, USA).

2. Clinical Presentation

We present the case of a 2-year-old spayed female Netherland dwarf rabbit. This rabbit was dewormed yearly with fenbendazole, (Panacur®, Merck Animal Health, Giralda Farms, Madison, WI, USA, 20 mg/kg) and twice a year with a 15 mg selamectin spot-on (Stronghold®, Zoetis, Belgium vaccinated yearly (Nobivac®Myxo-RHD, MSD, Kenilworth, NJ, USA), and was kept indoors with an owner residing in the Oeiras District, Portugal. At the age of 18 months, the patient was presented for annual vaccination. Physical examination at that point was unremarkable and the rabbit was clinically normal.

In February 2020, 2 months after the previous visit, the rabbit presented for anorexia, coprostasis and lethargy. The physical examination only revealed a painful condition as the rabbit exhibited bruxism (teeth grinding) after cranial abdominal palpation. The rest of the physical examination was unremarkable and the rabbit weighed 1580 g at the time. A complete blood count (CBC, detailed in Table 1) revealed leukopenia (2.53 K/μL) with heteropenia (0.71 K/μL) and thrombocytopenia (40 K/μL). Alanine aminotransferase (ALT) levels were elevated (240 U/L), indicating liver damage and radiographs showed a more radio-opaque cranial abdomen. The patient was admitted and started on standard intravenous fluid therapy with NaCl 0.9%, was administered buprenorphine (0.03 mg/kg IV TID) for analgesia and provided nutritional support (Oxbow's herbivore critical care), recovering appetite about 6 hours later.

Table 1. Summary of the rabbit haematological and biochemical parameters and respective reference values retrieved from Carpenter's 4th ed of the Exotic animal formulary [23] and Idexx's reference values of the ProCyte Dx* Haematology Analyzer (as of IDEXX VetLab* Station software version 4.48) and Catalyst one Biochemistry analyser, the equipment used in this study.

Haematology	February	March	June	Reference Values	
				Carpenter, 2018	Idexx Lab., 2017
Haematocrit (%)	30.9	36.3	37.6	30–50	29.4–40.9
Haemoglobin (g/dL)	10.7	11.8	13.3	8–17.5	9.8–13.2
Erythrocytes ($\times 10^6$/µL)	5.21	5.67	6.45	4–8	4.45–6.71
MCV (fL)	59.3	64.0	58.3	58–75	58.1–69.6
MCH (pg)	20.5	20.8	20.6	17.5–23.5	18.9–22.1
MCHC (g/dL)	34.6	32.5	35.4	29–37	31.6–33.6
Reticulocytes (%)	2.4	**5.4**	1.4	2–4	-
Reticulocytes (K/µL)	122.4	**306.7↑**	88.4	-	69.5–242.7
Platelets (10^3/µL)	**40↓**	370	**97↓**	290–650	219–521
WBC (10^3/µL)	**2.53↓**	5.15	**1.86↓**	5–12	4.54–10.22
Heterophils (%)	**28.1↓**	**28.8↓**	47.9	35–55	-
Lymphocytes (%)	**64.4↑**	60.0	41.9	25–60	-
Monocytes (%)	4.7	5.4	4.8	2–10	-
Eosinophils (%)	0.8	0.8	0.0	0–5	-
Basophils (%)	2.0	5.0	5.4	2–8	-
Heterophils (K/µL)	**0.71↓**	1.48	**0.89↓**	-	0.96–3.34
Lymphocytes (K/µL)	1.63	3.09	**0.78↓**	-	1.49–5.21
Monocytes (K/µL)	**0.12↓**	**0.28↓**	**0.09↓**	-	0.31–0.99
Eosinophils (K/µL)	**0.02↓**	**0.04↓**	**0.00↓**	-	0.05–2.12
Basophils (K/µL)	**0.05↓**	**0.26↓**	**0.10↓**	-	0.56–2.12
Biochemistry					
Glucose	**194↑**	-	**164↑**	75–150	75–145
Creatinine (CREA)	1.0	-	1.2	0.5–2.6	0.8–1.8
BUN	12	-	22	15–50	10–24
BUN/CREA	12	-	18	-	-
PHOS	-	-	3.7	2.3–6.9	1.2–4.9
CA	-	-	11.7	8–14.8	5.6–12.0
TP	6.6	-	**7.7↑**	5.4–7.5	5.5–7.2
ALB	4.4	-	4.4	2.5–5	2.7–4.6
GLOB	2.2	-	**3.3↑**	1.5–3.5	1.5–2.8
ALB/GLOB	2.0	-	1.3	-	-
ALT	**240↑**	-	**586↑**	14–80	31–53
ALKP	99	-	104	4–70	70–145
GGT	-	-	2	-	-
TBIL	-	-	0.9	0–0.75	0.3–0.8
CHOL	-	-	30	12–16	35–53

The values highlighted in bold represent the main deviations observed and the arrows indicate if the values are above (↑) or below (↓) the references.

Ultrasound revealed a slightly heterogeneous liver with a hypoechoic caudate lobe and a kidney stone considered meaningless for this case. There were no obvious signs of vascular compromise. However, shaving the abdomen for the ultrasound exam revealed a large haematoma that extended from the neck to forelimbs, thorax and upper-half of the abdomen. This was assumed to have originated from the jugular blood collection carried out in the previous day, despite the seemingly atraumatic puncture.

Throughout the 5th day of hospitalisation, the patient maintained a pattern of cutaneous haemorrhagic dyscrasia, with vascular fragility, easy bruising and requiring the need for prolonged compression of puncture sites. Coagulation tests were carried out and both prothrombin time (PT) and activated partial thromboplastin time (aPTT) were prolonged, at 25 and 195 s, respectively.

A CT scan showed no signs of hepatic abnormalities or vascular compromise. The patient was started on injectable vitamin K1 (10 mg/kg SC SID) and kept on buprenorphine (0.03 mg/kg SC TID) and IV NaCl 0.9% at a rate of 5mL/h. Bruising was fully reabsorbed by the 8th day and the patient was sent home. Before release, the physical exam was unremarkable. The rabbit was bright and alert and had fully recovered his appetite, weighting at the time 1500 g. No more blood collections were possible due to lack of venous accesses, secondary to multiple hematomas and bruising in the previous blood collection sites. Two weeks later, in March 2020, follow up blood tests were performed, including a CBC. The revaluation hemogram was unremarkable, as reticulocytosis was considered a normal finding given the events in the previous hospitalisation. No other clinical follow up could be performed due to financial limitations and lockdown policies associated with the COVID-19 world pandemic.

In June 2020, the rabbit was readmitted due to anorexia, lethargy, coprostasis and abdominal discomfort. The rabbit had lost 80 g, weighting then 1420 g, and for the first time, mildly hyperthermia (39.4 °C) was registered. The blood panel revealed severe leukopenia (1.86 K/µL) associated with heteropenia (0.89 K/µL) and lymphopenia (0.78 K/µL). Thrombocytopenia (97 K/µL) was also observed. ALT was then 586 U/L and there was also the elevation of total bilirubin (0.9 mg/dL) and total proteins (7.7 g/dL) due to hyperglobulinemia (3.3 g/dL) (Table 1). The blood draw resulted once again in cutaneous haemorrhagic dyscrasia and therefore the rabbit was started on vitamin K1 (10 mg/kg SC BID), enrofloxacin (5 mg/kg SC SID), buprenorphine (0.03 mg/kg SC TID), metoclopramide (0.5 mg/kg SC BID), lactulose (0.5 mL/kg PO BID) and aggressive fluid therapy. Ultrasound was compatible with severe hepatitis and peri-lobular peritonitis (Figure 2), with mesenteric reactivity and free fluid in the abdomen. The rabbit was in the hospital for 3 days showing no signs of improvement. The temperature kept increasing reaching a peak of 40.2 °C on the second day of hospitalisation, after which it started dropping and the rabbit became progressively hypothermic. On the 3rd day of hospitalization, the temperature dropped to 36.9 °C, despite the active heating efforts. The rabbit became lethargic, jaundiced and developed vertical nystagmus. The death occurred about 6 h after the onset of these symptoms.
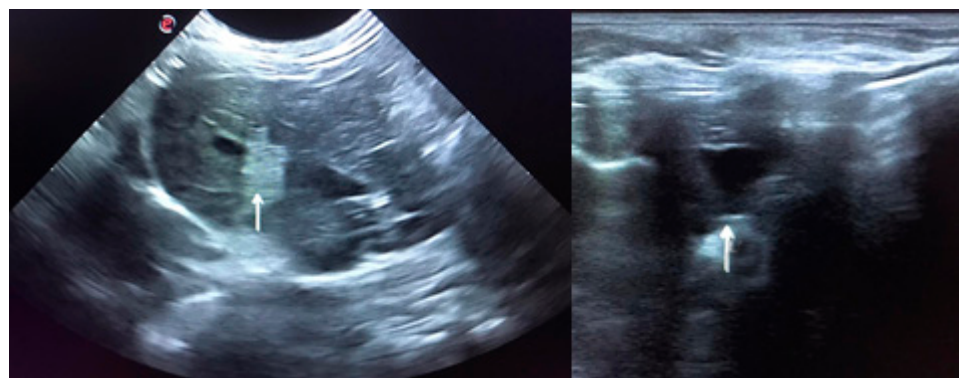
Figure 2. Ultrasound is compatible with hepatitis. The image on the left shows peri-lobular mesenteric reactivity (arrow). On the right, free fluid in the abdomen is visible (arrow).

Throughout the clinical evolution of this case, several differential diagnoses were considered. These included hepatic lipidosis, hepatic coccidiosis, liver lobe torsion, bacterial, fungal, and parasitic hepatitis, Encephalitozoon cuniculi infection, Tyzzer's disease and neoplasia. All these possibilities were excluded by the rabbit's clinical history, semestral coprology results and deworming history, along with the blood works, radiographs, ultrasounds, CT scan and post mortem data.

The necropsy findings reinforced that the inflammatory and/or infectious process was the most likely differential diagnosis for this patient.

3. Material and Methods

3.1. Necropsy and Histopathology

The necropsy was performed according to routine procedures, and samples were collected for bacteriology (liver, spleen and lung), histopathology (liver, spleen, stomach, small intestine, pancreas and kidney) and virology (liver). All analyses followed routine procedures.

Histopathology fragments were fixed in 10% neutral buffered formalin, routinely paraffin-embedded, sectioned at 4 μm, and stained with Haematoxylin and Eosin (H&E).

3.2. Molecular Analysis

For nucleic acid extraction, a fresh sample of liver was homogenized at 10% (w/v) with phosphate-buffered saline (PBS) and clarified at 3000 g for 5 min. Total nucleic acids were extracted from 200 μL of the clarified supernatants, using the MagAttract 96 cador Pathogen Kit (Qiagen, Hilden, Germany) in a BioSprint 96 nucleic acid extractor (Qiagen, Hilden, Germany), according to the manufacturer's protocol.

The rabbit was tested for rabbit haemorrhagic disease virus (RHDV) by conventional PCR [24] and rabbit haemorrhagic disease virus 2 (RHDV2) and myxomavirus (MYXV) by real-time PCR [25,26]. Amplifications were carried out in a Bio-Rad CFX96™ Thermal Cycler (Bio-Rad Laboratories Srl, Redmond, WA, USA), using the One-Step RT-PCR kit (Qiagen, Hilden, Germany) for RHDV2, and the NZYTaq II 2x Colourless Master Mix (Nzytech, Lisbon, Portugal) for MYXV.

cDNA was synthesised with the SuperScript™ IV First-Strand Synthesis System (InVitrogen, Carlsbad, CA, USA) according to the manufacturer's recommendations, using either oligo(dT)12-18 and random hexamers. Amplification of full VP60 gene and partial RdRp gene was achieved using primers, kits and protocols available in appendix Table S1.

The PCR products were visualised in 2% horizontal electrophoresis agarose gel, purified using 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, Foster City, CA, USA). Nucleotide sequences were analysed and assembled into consensus sequences using the Seqscape Software v2.7 (Applied Biosystems, Foster City, CA, USA)., The final 2176 nucleotide sequence including the VP60 complete gene (1740 nt-long) and a 436 nt-long region of the RdRp gene, was deposited in GenBank database and given the accession number MT829254.

3.3. Phylogenetic Analysis

To further investigate the relation of this strain to other recombinants characterised previously in Portugal [8], a phylogenetic analysis was carried out by Maximum Likelihood (ML) resourcing to the R software (R Development Core Team, 2009) [27]. Three types of multiple sequence alignments (msa) were used for phylogenetic inference, encompassing a 436 nt region within the RdRp gene, the complete 1740 nt vp60 gene sequences, and the 2176 nt long sequence comprising the two regions mentioned.

Multiple sequence alignments (msa) were generated by MUSCLE through the R software (R Development Core Team, 2009). For each alignment, the appropriate substitution model for ML analysis was determined using the function modeltest. For RdRp tree, the Hasengawa–Kishino–Yano (HKY) model [28] an allowance for the incorporation of invariant sites (I) (HKY+I), showed the lower AIC value and was used to infer phylogenetic relationships. For the ML phylogenetic tree based on the full vp60 gene (1740 nt long) the General Time Reversible (GTR) model [29] with a discrete gamma distribution (+G), (GTR+G) showed the lower AIC value was used to infer phylogenetic relationships. For the ML phylogenetic tree based on a 2176 nt long sequence comprising the terminal 436 nt long region within the RdRp gene and the complete vp60 gene the General Time Reversible (GTR) model [29] with a discrete gamma distribution (+G) and/or an allowance for the incorporation of invariant sites (+I) (GTR+G+I) showed the lower AIC value and was used to infer phylogenetic relationships.

4. Results

4.1. Necropsy and Histopathology

The necropsy revealed liver congestion and marked lobular pattern, presence of a small amount of free peritoneal fluid and congested lungs with haemorrhagic foci. Both kidneys showed areas of surface retraction accounting for about 20% of the total surface.

The following microscopic lesions were observed: **Liver**: severe generalized perilobular haemorrhagic necrosis (Figures 3 and 4). Discrete infiltration by mononucleated inflammatory cells, mainly macrophages and lymphocytes, around portal triads. Fine brown pigment in the cells of the portal bile ducts. **Spleen**: diffuse necrosis of the entire red pulp revealed by deposition of fibrinoid acidophilic material drawing serpiginous patterns in the parenchyma (Figure 5). The regular presence of lymphoid follicles around central arterioles. **Pancreas**: interlobular oedema and necrosis of adipocytes, both intralobular and interlobular. No changes were present in the secretory cells. **Stomach**: no significant changes were observed. **Small intestine**: Necrotic enteritis, particularly in the duodenum, with loss of villi and deposition of fibrin in the proximal mucosa (Figure 6). **Kidneys**: the areas of surface retraction in both kidneys corresponded to segmental fibrosis affecting cortex and medulla. In these areas, there was a loss of tubules and glomeruli, which were moderately congested. No microbial agents were identified in any organ and the results of the microbiological analysis were also negative.
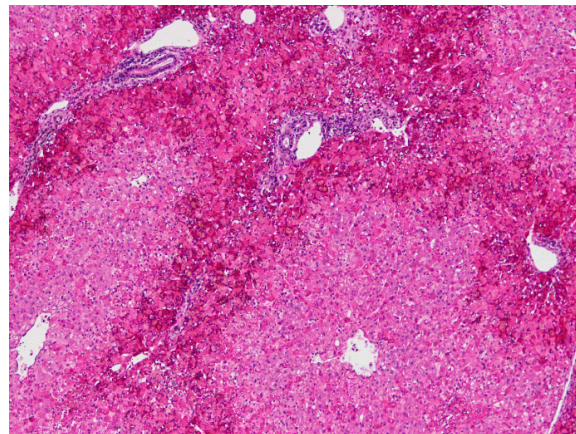
Figure 3. Liver. Severe haemorrhagic necrosis consistently affects perilobular areas or acinar zone 1, conspicuous due to the bright red colour close to the portal areas (H&E, 40×).
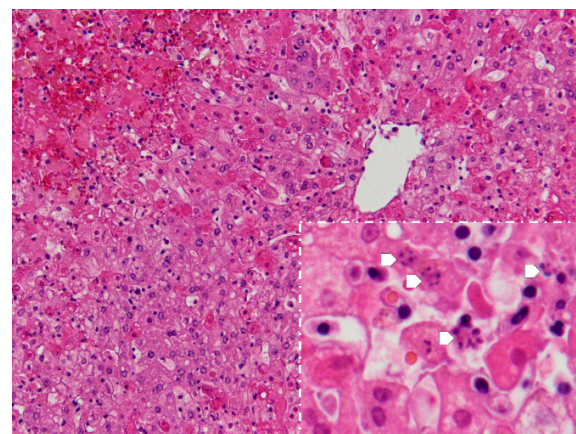
Figure 4. Liver. Apart from perilobular haemorrhagic necrosis, single-cell necrosis is present in dispersed hepatocytes in acinar zones 2 and 3, which surround the periacinar vein in the upper right. Inset-magnification of the acinar zones 2 and 3, showing various cells with the fragmentation of the nucleus-karyorrhexis (arrows) (H&E, 100×, inset 400×).
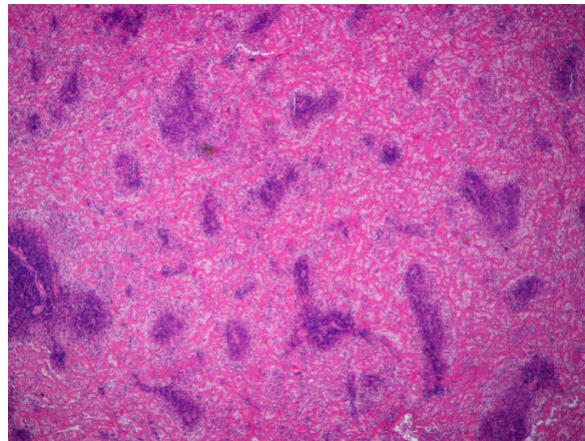
Figure 5. Spleen. Severe diffuse fibrinoid necrosis of the red pulp. The eosinophilic material fibrinoid material is very abundant between the lymphoid tissue that surrounds blood vessels (H&E, 40×).
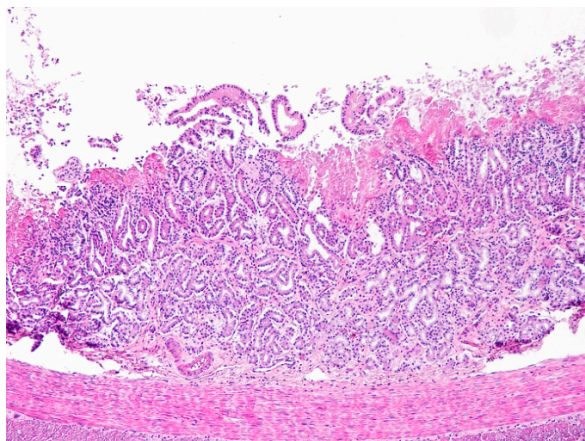
Figure 6. Duodenum. Necrotic enteritis affecting the upper mucosa. Note the absence of villi and the eosinophilic deposits of fibrin in the upper mucosa. The intestinal glands are disorganized and the inflammatory infiltrate of the mucosal lamina propria by mononuclear cells (lymphocytes mostly) is scant (H&E, 100×).

4.2. Virology

Investigations for MYXV and RHDV (genotypes GI.1b (former G1) to GI.1a (former RHDVa or G6)) [19] were negative. However, the RT-qPCR specific for RHDV2 revealed a high viral load in the liver (approximately 9.0×10^{11} copies of RHDV2 RNA per mg of liver), higher than the usual viral load found in infected rabbit liver, including both vaccinated and unvaccinated rabbits [30]. The amplification of the RdRp partial gene and VP60 complete gene was successful, generating amplicons within the expected sizes (available in access number MT829254).

4.3. Phylogenetic Analysis

BLAST analysis of VP60 nucleotide sequence showed 96.88% similarity with RHDV2 sequences characterised earlier in 2013 from mainland Portugal, namely with three strains (KF44962, KF44963 and KF44964) collected from wild rabbits originating from the south region, Alentejo and Algarve. BLAST analysis of the partial RdRp gene showed 96.56% similarity with a recombinant RHDV2/NP1 strain (MG763952) collected from a wild rabbit from the north of mainland Portugal in 2015.

BLAST analysis of the 2176 nt long sequence revealed higher similarity with sequence KF442964, obtained from a wild rabbit from South of mainland Portugal sampled in 2013.

The tree based on the 3' end sequence of RpRd gene (fragment 436 bp), confirmed that the RHDV2 strain from the dwarf rabbit shared high similarity with the homologous region of the RdRp from other RHDV2 recombinants classified as GI.4 (NP1)/RHDV2 (Figure 7A). The VP60 gene base tree and phylogenetic analysis including the complete fragment showed that the dwarf sequence RHDV2 is in an isolated branch and does not group within the defined clusters (Figure 7B) and (Figure 7C).
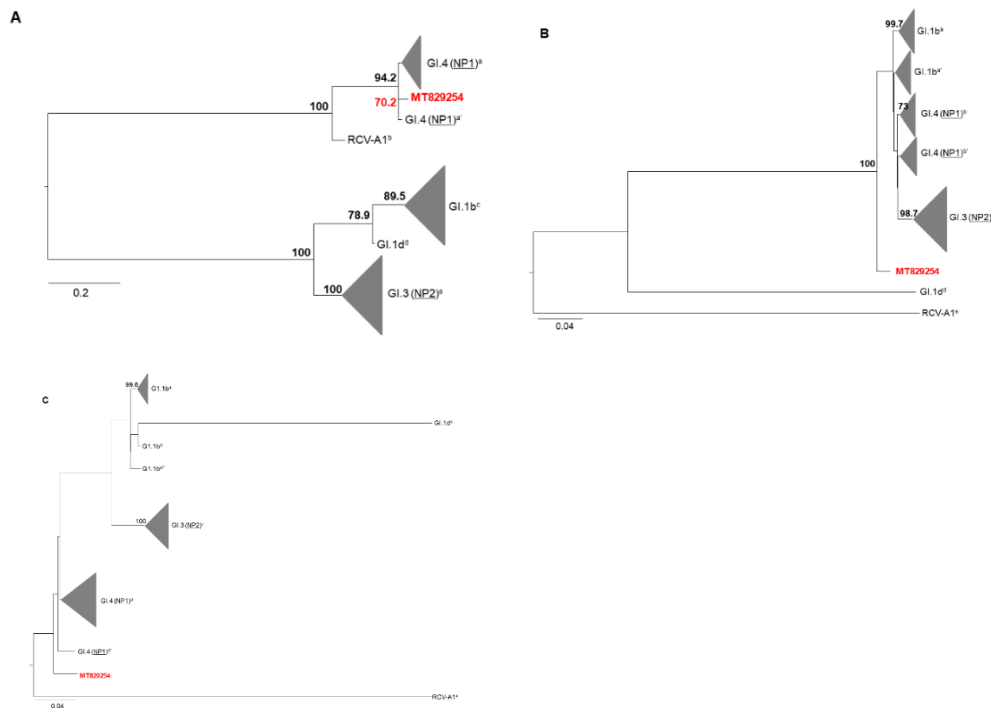
Figure 7. Phylogenetic analysis. (**A**) Maximum likelihood analysis using 14 RdRp gene partial (436 nt) nucleotide sequences, namely GI.4 (NP1–non-pathogenic caliciviruses similar to RCV-A1 are the most likely donors of non-structural proteins) representatives (a-MG763946, MG763944, MG763954 and a'-MG763952), GI.1b (G1) representatives (c-MG763939, MG763938, MG763947 and MG763953), GI.3 (NP2-non-pathogenic caliciviruses similar to CBAnd1 are the most likely donors of non-structural proteins) representatives (e-MG763942, MG763949, MG763943 and MG763945; GI.1d (G3-G5) representatives (d-MH190418) and non-pathogenic rabbit calicivirus Australia 1 (RCV-A1) representatives (b-EU871528). (**B**) Maximum likelihood analysis using 14 complete VP60 gene nucleotide sequences including, GI.1b representatives (a-MG763939 and MG763938 and a'-MG763953 and MG763947), GI.4 (NP1) representatives (b-MG763952 and MG763954 and b'-MG763946 and MG763944), GI.3 (NP2) representatives (c-MG763949, MG763942, MG763945 and MG763943; GI.1d representatives (d-MH190418) and RCVA-A1 representatives (e-EU871528). (**C**) Maximum likelihood analysis using 14 sequences comprising the terminus of the RdRp gene and the complete VP60 gene, namely GI.1b representatives (a-MG763938 and MG763939, a'-MG763947, and a''-MG763953), GI.3 (NP2) representatives (c-MG763949, MG763942, MG763945 and MG763943), GI.4 (NP1) representatives (d-MG763954, MG763946, MG763944 and d'-MG763952) GI.1d representatives (b-MH190418) and RCVA1 representatives (e-EU871528). The nomenclature used is in accordance with Silvério et al., 2018 [8], Abrantes et al., 2020 [31]. Designations only used by Silvério et al., 2018 are underlined in the phylogenetic trees.

Robustness of the tree nodes was assessed by bootstrapping 1000 times. Only bootstrap (BS) values greater or equal to 70 are shown. The graphical representation and edition of the phylogenetic trees were performed with FigTree v1.3.1 (http://tree.bio.ed.ac.uk/software/figtree/).

5. Discussion

Rabbit haemorrhagic disease affects both domestic and wild rabbits causing a systemic disease usually with a lethal outcome. After its emergence, RHDV2 strains evolved quickly, with some variation of amino acids in the capsid protein but maintaining the original

RHDV2 antigenic profile. Despite this, several RHDV2 recombinants containing the non-structural protein genes of other rabbit lagoviruses (such as GI.1b (G1) and GI.4 or GI.3 (NP-CV)) have been identified in Europe [8]. These include the structural protein (VP60 and VP10) encoding genes of RHDV2 combined with the non-structural protein-encoding genes of GI.1b (RHDV genogroup G1 strain), non-pathogenic rabbit caliciviruses Australia 1 (RCV-A1)-like viruses (GI.4) or other non-pathogenic lagoviruses (GI.3) [8,32,33].

Here we describe an RHDV2 infection in a 2-year-old dwarf rabbit that could have developed an atypical form of RHD before the eventual fatality, four months after the presentation of first signs compatible with the disease. The conclusive diagnosis of RHDV2 was only made post-mortem, making it impossible to claim that the clinical picture observed between February and June was due to RHDV2 infection. However, the clinical signs and the results of the diagnostic investigations (ultrasound hepatic and peri-hepatic changes, an elevated marker of liver injury-ALT, raised total bilirubin and jaundice, hyperglobulinemia and poor coagulation (namely severe subcutaneous haemorrhage and increased prothrombin time (PT) and activated partial thromboplastin time (aPTT))) are all compatible with Rabbit Haemorrhagic Disease. Moreover, no other possible cause for the clinical signs was found. Most of the previous clinical signs, along with a normal hemogram, a leukogram revealing leukopenia and thrombocytopenia have been previously associated with rabbit haemorrhagic disease in a review by Bonvehí et al., 2019 [34].

The atypical development of the clinical course, much longer than the usual, could have resulted from deficient cross-immunity conferred by RHDV vaccination, leading to a subacute disease.

Despite RHDV vaccines do not confer full protection against RHDV2, rabbits vaccinated with RDHV cannot be assumed as immunologically naive against RHDV2 as non-vaccinated animals. The cross-immunity between RHDV and RHDV2 is reported as deficient but not as inexistent [9]. Therefore, it is expected that RHDV-vaccinated animals do not show a primary immune response to RHDV2 infection, since cross-reactive responses (RHDV-RHDV2) to particular epitopes may be beneficial to the protective response, even if deficient.

In fact, the studies on RHDV/RHDV2 cross-immunity are limited to a few strains that emerged soon after 2010. For this reason, the current circulating RHDV2 strains may have different pathogenic characteristics that may explain an unusual presentation in RHDV vaccinated in rabbits. The vaccine used contains a recombinant myxoma virus that expresses the VP60 protein of RHDV, thus can never be responsible for inducing RHD in rabbits. Also, the strain isolated in this animal (RHDV2) is completely different from the one contained in the vaccine (RHDV).

The origin of this infection is unknown. However, in Oeiras district, there are a few populations of wild rabbit that may have been a source of infection for this animal through arthropod vectors. Infection in a hospital context is not probable, considering that there was no case of RHD in that hospital in the last year and that strict disinfection guidelines for spaces and equipment are followed.

The virus identified in this rabbit was confirmed to be a recombinant strain, with the structural backbone of RHDV2 and the non-structural genes of non-pathogenic RCV-A1 strains (GI.4). The clinical course was suggestive, but non-pathognomonic, of rabbit haemorrhagic disease. Post-mortem lesions identified in the liver, spleen and intestine are compatible with an acute clinical course of the disease with fatal outcome. Kidney lesions, on the contrary, are typical of a chronic process, but their severity was not enough to consider renal insufficiency as the cause for the clinical signs.

No studies are evaluating a longer clinical course of the disease caused by RHDV2 recombinant strains such as the one detected in this rabbit. Further studies are necessary to better understand the possibility of different genotypes generating different clinical courses of disease. This case is an important warning to all veterinarians drawing attention to the fact that RHD is an important differential diagnosis to be considered in some clinical

settings, and that it is critical to revise and update the vaccination programs towards RHDV2 infection.

References

1. Le Pendu, J.; Abrantes, J.; Bertagnoli, S.; Guitton, J.-S.; Le Gall-Reculé, G.; Lopes, A.M.; Marchandeau, S.; Alda, F.; Almeida, T.; Célio, A.P.; et al. Proposal for a unified classification system and nomenclature of lagoviruses. *J. Gen. Virol.* **2017**, *98*, 1658–1666. [CrossRef] [PubMed]
2. Lees, A.C.; Bell, D.J. A conservation paradox for the 21st century: The European wild rabbit *Oryctolagus cuniculus*, an invasive alien and an endangered native species. *Mamm. Rev.* **2008**, *38*, 304–320. [CrossRef]
3. Neimanis, A.; Larsson Pettersson, U.; Huang, N.; Gavier-Widén, D.; Strive, T. Elucidation of the pathology and tissue distribution of Lagovirus europaeus GI.2/RHDV2 (rabbit haemorrhagic disease virus 2) in young and adult rabbits (*Oryctolagus cuniculus*). *Vet. Res.* **2018**, *49*, 46. [CrossRef] [PubMed]
4. OIE, 2019: Rabbit Haemorrhagic Disease (Technical Disease Card). 1–7. Available online: https://www.oie.int/fileadmin/Home/eng/Animal_Health_in_the_World/docs/pdf/Disease_cards/RHD.pdf (accessed on 3 October 2020).
5. Ramiro-Ibáñez, F.; Martín-Alonso, J.M.; García Palencia, P.; Parra, F.; Alonso, C. Macrophage tropism of rabbit hemorrhagic disease virus is associated with vascular pathology. *Virus Res.* **1999**, *60*, 21–28.
6. Villafuerte, R.; Calvete, C.; Blanco, J.C.; Lucientes, J. Incidence of viral hemorrhagic disease in wild rabbit populations in Spain. *Mammalia* **1995**, 651–660. [CrossRef]
7. Le Gall-Recule, G.; Zwingelstein, F.; Boucher, S.; Le Normand, B.; Plassiart, G.; Portejoie, Y.; Decors, A.; Bertagnoli, S.; Guerin, J.-L.; Marchandeau, S. Detection of a new variant of rabbit haemorrhagic disease virus in France. *Vet. Rec.* **2011**, *168*, 137–138. [CrossRef]
8. Silvério, D.; Lopes, A.M.; Melo-ferreira, J.; Magalhaes, M.J.; Ponterroso, P.; Serronha, A.; Maio, E.; Alves, P.C.; Esteves, P.J.; Abrantes, J. Insights into the evolution of the new variant rabbit haemorrhagic disease virus (GI. 2) and the identification of novel recombinant strains. *Transbound. Emerg. Dis.* **2018**, *65*, 983–992. [CrossRef]
9. Le Gall-Reculé, G.; Lavazza, A.; Marchandeau, S.; Bertagnoli, S.; Zwingelstein, F.; Cavadini, P.; Martinelli, N.; Lombardi, G.; Guérin, J.L.; Lemaitre, E.; et al. Emergence of a new lagovirus related to rabbit haemorrhagic disease virus. *Vet. Res.* **2013**, *44*, 81. [CrossRef]
10. Harcourt-Brown, N.; Silkstone, M.; Whitbread, T.J.; Harcourt-Brown, F.M. RHDV2 epidemic in UK pet rabbits. Part 1: Clinical features, gross post mortem and histopathological findings. *J. Small Anim. Pract.* **2020**, *61*, 419–427. [CrossRef]
11. Abade dos Santos, F.A.; Carvalho, C.; Nuno, O.; Correia, J.J.; Henriques, M.; Peleteiro, M.C.; Fevereiro, M.; Duarte, M.D. Detection of rabbit Haemorrhagic disease virus 2 during the wild rabbit (*Oryctolagus cuniculus*) eradication from the Berlengas archipelago, Portugal. *BMC Vet. Res.* **2017**, *13*, 336. [CrossRef]
12. Abade dos Santos, F.A. *Quadro Anatomo-Histopatológico e Diagnóstico Molecular da Doença Hemorrágica Viral em Coelho-Bravo*; Faculty of Veterinary Medicine, University of Lisbon: Lisbon, Portugal, 2018.
13. Barthold, W.S.; Griffey, S.M.; Percy, D.; Rabbit, H. *Pathology of Laboratory Rodents and Rabbits*; Wiley-Blackwell: Hoboken, NJ, USA, 2016; ISBN 9781118824245.

14. Trzeciak-Ryczek, A.; Tokarz-Deptuła, B.; Deptuła, W. The importance of liver lesions and changes to biochemical and coagulation factors in the pathogenesis of RHD. *Acta Biochim. Pol.* **2015**, *62*, 169–171. [CrossRef] [PubMed]

15. Plassiart, G.; Guelfi, J.-F.; Ganiere, J.-P.; Wang, B.; Andre-Fontaine, G.; Wyers, M. Hematological Parameters and Visceral Lesions Relationships in Rabbit Viral Hemorrhagic Disease. *J. Vet. Med. Ser. B* **1992**, *39*, 443–453. [CrossRef] [PubMed]

16. Park, J.H.; Lee, Y.S.; Itakura, C. Fibrin(ogen)-related antigens in rabbits experimentally infected with rabbit haemorrhagic disease virus. *Res. Vet. Sci.* **1997**, *63*, 123–127. [CrossRef]

17. Ueda, K.; Park, J.H.; Ochiai, K.; Itakura, C. Disseminated intravascular coagulation (DIC) in rabbit haemorrhagic disease. *Jpn. J. Vet. Res.* **1992**, *40*, 133–141. [PubMed]

18. Rocchi, M.S.; Dagleish, M.P. Diagnosis and prevention of rabbit viral haemorrhagic disease 2. *In Pract.* **2018**, *40*, 11–16. [CrossRef]

19. Soliman, M.; Rhaman, M.; Samy, M.; Mehana, O.; Nasef, S. Molecular, Clinical and Pathological Studies on Viral Rabbit Hemorrhagic Disease. *Alex. J. Vet. Sci.* **2016**, *48*, 20. [CrossRef]

20. Duarte, M.D.; Carvalho, C.L.; Abade dos Santos, F.A.; Gomes, J.; Alves, P.C.; Esteves, P.J.; Abrantes, J.; Lopes, A.M.; Lopes, P.M.; Serronha, A.; et al. +Coelho: Avaliação Ecossanitária das Populações Naturais de Coelho Bravo Visando o Controlo da Doença Hemorrágica Viral. Master's Thesis, University of Lisbon, Lisboa, Portugal, 2018.

21. Montbrau, C.; Padrell, M.; Ruiz, M.C. Efficacy and Safety of a New Inactivated Vaccine against the Rabbit Haemorrhagic Disease Virus 2-Like Variant (RHDV-2). 2012, Volume 1984. Available online: https://www.hipra.com/wcm/connect/hipra/d0be8fc3-788a-4910-a47e-64709166ad1c/Efficacy+and+safety+of+a+new+inactivated+vaccine+against+RHDV-2.pdf?MOD=AJPERES&CACHEID=ROOTWORKSPACE.Z18_GG50HI40O8ABD0Q8OC940F2000-d0be8fc3-788a-4910-a47e-64709166ad1c-lAmNnR1 (accessed on 25 September 2020).

22. Office International des Epizooties. OIE Rabbit Haemorrhagic Disease. In *OIE Terrestrial Manual 2018*; Office International des Epizooties: Paris, France, 2018; pp. 1389–1406. Available online: www.oie.int (accessed on 15 September 2020).

23. Fisher, P.; Graham, J. Chapter 10—Rabbits. In *Exotic Animal Formulary*, 5th ed.; Carpenter, J.W., Marion, C.J., Eds.; Elsevier Saunders: St. Louis, MI, USA, 2018; pp. 494–531. ISBN 978-0-323-44450-7.

24. Tham, K.M.; Barnes, S.M.; Hunter, S.N. Polymerase chain reaction amplification and gene sequence analysis of a calicivirus from a feral rabbit. *Virus Genes* **1999**, *18*, 235–242. [CrossRef]

25. Duarte, M.D.; Carvalho, C.L.; Barros, S.C.; Henriques, A.M.; Ramos, F.; Fagulha, T.; Luís, T.; Duarte, E.L.; Fevereiro, M. A real time Taqman RT-PCR for the detection of rabbit hemorrhagic disease virus 2 (RHDV2). *J. Virol. Methods* **2015**, *219*, 90–95. [CrossRef]

26. Duarte, M.D.; Barros, S.C.; Henriques, A.M.; Fagulha, M.T.; Ramos, F.; Luís, T.; Fevereiro, M. Development and validation of a real time PCR for the detection of myxoma virus based on the diploid gene M000. 5L/R. *J. Virol. Methods* **2014**, *196*, 219–224. [CrossRef]

27. R Development Core Team R. *A Language and Environment for Statistical Computing*; R Found. Stat. Comput.: Vienna, Austria, 2008; ISBN 3-900051-07-0.

28. Hasegawa, M.; Kishino, H.; Yano, T. Dating of the human-ape splitting by a molecular clock of mitochondrial DNA. *J. Mol. Evol.* **1985**, *22*, 160–174. [CrossRef]

29. Tavaré, S.; Miura, R.M. Lectures on mathematics in the life sciences. *Am. Math. Soc.* **1986**, 57–86.

30. Carvalho, C.L.; Duarte, E.L.; Monteiro, M.; Botelho, A.; Albuquerque, T.; Fevereiro, M.; Henriques, A.M.; Barros, S.S.; Duarte, M.D. Challenges in the rabbit haemorrhagic disease 2 (RHDV2) molecular diagnosis of vaccinated rabbits. *Vet. Microbiol.* **2017**, *198*, 43–50. [CrossRef] [PubMed]

31. Abrantes, J.; Droillard, C.; Lopes, A.M.; Lemaitre, E.; Lucas, P.; Blanchard, Y.; Marchandeau, S.; Esteves, P.J.; Le Gall-Reculé, G. Recombination at the emergence of the pathogenic rabbit haemorrhagic disease virus Lagovirus europaeus/GI.2. *Sci. Rep.* **2020**, *10*, 1–11. [CrossRef] [PubMed]

32. Lopes, A.M.; Blanco-Aguiar, J.; Martín-Alonso, A.; Leitão, M.; Foronda, P.; Mendes, M.; Gonçalves, D.; Abrantes, J.; Esteves, P.J. Full genome sequences are key to disclose RHDV2 emergence in the Macaronesian islands. *Virus Genes* **2018**, *54*, 1–4. [CrossRef]

33. Dalton, K.P.; Arnal, J.L.; Benito, A.A.; Chacón, G.; Martín Alonso, J.M.; Parra, F. Conventional and real time RT-PCR assays for the detection and differentiation of variant rabbit hemorrhagic disease virus (RHDVb) and its recombinants. *J. Virol. Methods* **2018**, *251*, 118–122. [CrossRef]

34. Bonvehí, C.; Ardiaca, M.; Montesinos, A.; Juan, C.; Gómez, A.; Teso, B.; Barbero, S.; Ekei, S. Clinicopathologic findings of naturally occurring Rabbit Hemorrhagic Disease Virus 2 infection in pet rabbits. *Vet. Clin. Pathol.* **2019**, *48*, 89–95. [CrossRef]

Scientific publication 8

Myxoma virus and rabbit haemorrhagic disease virus 2 coinfection in a European wild rabbit (Oryctolagus cuniculus algirus), Portugal

■ ■

Myxomatosis and rabbit haemorragic disease co-exist in the rabbit populations since 1986. However, coinfection by these two viruses was considered for a long time a rare or impossible event.
In early 2019, we detected a case of co-infection by RHDV2 and myxoma virus, with both lesional compatible pathological patterns, demonstrating that, although rarely, rabbits can be simultaneously infected by the two viruses.

WILDLIFE

Myxoma virus and rabbit haemorrhagic disease virus 2 coinfection in a European wild rabbit (*Oryctolagus cuniculus algirus*), Portugal

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SUMMARY

Myxoma virus (MYXV) and rabbit haemorrhagic disease virus 2 (RHDV2) are two major pathogens that affect the European rabbit (*Oryctolagus cuniculus*). Between August 2017 and August 2019, 1166 wild rabbits (971 legally hunted and 195 found dead) were tested by PCR-based methods for MYXV and RHDV2 within the scope of an ongoing surveillance programme on wild leporids in Portugal. Despite never having been reported before and being considered a rare event, coinfection by RHDV2 and MYXV was detected in one juvenile wild rabbit found dead in the Évora district located in Alentejo. The relative frequency of coinfection in the group of diseased rabbits (found dead in the field) was 0.52 per cent (1/195). The positivity percentage of each single virus was much higher, namely, 14.36 per cent (28/195) for MYXV and 55.38 per cent (108/195) for RHDV2, within the 2 years of sample collection considered.

BACKGROUND

Myxoma virus (MYXV) and rabbit haemorrhagic disease virus 2 (RHDV2) are the two major pathogen threats for the European rabbit (*Oryctolagus cuniculus*).[1 2]

MYXV is a large double-stranded DNA *Leporipoxvirus* of the Poxviridae family.[3] In the European rabbit, it causes myxomatosis, a systemic and usually lethal disease[4] endemic in Iberia since 1953.[5] The disease still downregulates the wild rabbit populations, affecting mostly newborn and juvenile rabbits.[2]

RHDV2, a *Lagovirus* of the Caliciviridae family, emerged in France in 2010[6] and induces an acute, highly contagious and often lethal systemic disease in wild and domestic rabbits.[1 6] RHDV2 rapidly replaced the former rabbit haemorrhagic disease virus (RHDV),[7–9] first reported in 1984 in China.[10] Soon after their emergences, nearly 24 years apart, both viruses quickly spread throughout Europe,[11 12] causing high economic losses in the industry[6 12] and high mortality rates in wild rabbit populations.[1 12]

Serological surveys have demonstrated the concomitant presence of MYXV and RHDV/RHDV2 antibodies in wild rabbits.[13–15] Intriguingly, coinfections by these viruses were never reported in any of the many countries affected by the two diseases and were therefore considered rare or unexpected events.[16]

This study reports a coinfection event by MYXV and RHDV2 in a juvenile wild rabbit (*O cuniculus algirus*) found dead in Portugal.

CASE PRESENTATION

A male juvenile wild rabbit from the Évora district, Alentejo region, South of mainland Portugal, was found dead in the field on 20 February 2019. The animal was tested within the scope of an ongoing national surveillance programme on wild leporids (dispatch 4757/17, 31 May, Portuguese Ministry of Agriculture).

INVESTIGATIONS

Postmortem examination and histopathology

Necropsy of this juvenile wild rabbit was carried out at the National Reference Laboratory for Animal Diseases. During the necropsy, the animal presented poor body condition and skin lesions in the eyelids, ears, upper lips and genitals, suggestive of the nodular form of myxomatosis. Hepatic discoloration and multifocal congestion of the lungs, characteristic of rabbit haemorrhagic disease (RHD), were also observed.

For histopathological examination, skin samples were fixed in 10 per cent buffered formalin and embedded in paraffin using standard procedures. Five micrometre-thick sections were stained with H&E and examined using light microscopy.[17]

Histopathological examination of the eyelids and the upper lips showed typical myxoid tumours (myxomas) (figure 1).

Virological examination

Liver, spleen and lung samples were tested for the presence of RHDV, RHDV2 and MYXV by the PCR-based methods detailed further below. Skin lesions were also used for MYXV investigation.

For the virological screening, each tissue sample was homogenised with phosphate-buffered saline to a final concentration of 20 per cent (w/v) and clarified at $3000\,g$ for 5 minutes. Total DNA and RNA were extracted from $200\,\mu l$ 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.

Samples were tested for RHDV2 RNA by the RT-quantitative PCR (qPCR) by Duarte *et al*[18]
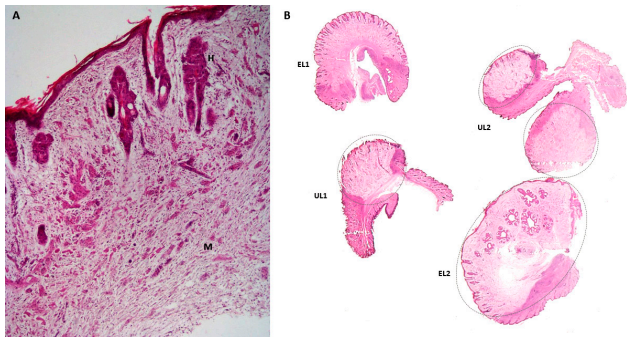
Figure 1 (A) Cross section of the eyelid showing the replacement of dermis by M and moderate H. H&E staining (×40 magnification). (B) Cross sections of the EL (EL1 and EL2) and ULs (UL1 and UL2) showing M nodules, pointed by dashed grey circles. H&E staining, original magnification. EL, eyelid; H, hyperplasia of the epidermis; M, myxoid tissue; UL, upper lip.

referred in the World Organisation for Animal Health (OIE) manual (https://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/3.06.02_RHD.pdf), using the One-Step RT-PCR Kit (Qiagen). The presence of MYXV DNA was investigated by the qPCR by Duarte et al,[19] also recommended in the OIE manual (https://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/3.06.01_MYXO.pdf), using the FastStart TaqMan Probe Master Kit (Roche, Roche Diagnostics GmbH, Manheim, Germany). For the real-time systems described, undetectable Cq or Cq values of >38.00 were considered negative. Positive controls were included in each reaction. Negative controls contained PCR-grade water.

Both MYXV DNA and RHDV2 RNA were detected in the liver, spleen and lungs of the juvenile wild rabbit. For RHDV2, as expected, the viral loads found in a pool of liver and spleen (9.09×10^8 copies/mg) were higher than those in the lungs (7.37×10^7 copies /mg).

MYXV DNA was detected in a skin lesion, where higher viral loads (2.49×10^7 copies/mg) were found, when compared with a pool of liver and spleen (6.87×10^5 copies/mg) or lungs (7.47×10^5 copies/mg) (figure 2). The lower viral loads detected in the internal organs suggest that MYXV replicates to low titres in the liver and lungs or may represent the contamination of organs with blood during the viraemic phase.

To rule out any contamination, samples from the juvenile wild rabbit were extracted *de novo* and retested, generating similar results.
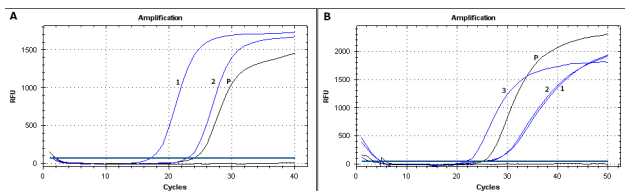
Figure 2 Amplification curves of (A) RHDV2 detection in the liver and spleen (1) (Cq value of 17.14) and lungs (2) (Cq value of 22.97), and (B) MYXV detection in the liver and spleen (1) (Cq value of 29.09), lungs (2) (Cq value of 28.98) and skin (3) (Cq value of 23.37). The negative controls (without nucleic acid) are the flat black lines seen below the thresholds. Cq, quantification cycle; P, positive control; RHDV2, rabbit haemorrhagic disease virus 2; MYXV, myxoma virus; RFU, Relative Fluorescence Units,

Screening for the classical strains of RHDV was performed by conventional PCR with primers RC-9 and RC-10[20] using the One-Step RT-PCR Kit (Qiagen). As expected, since the classical strains no longer circulate, the animal tested negative.

The full RHDV2 VP60 gene was amplified using the pairs of primers 27F[21] and 986R[22] and 717F[22] and 10R.[20] The fragment was excised from agarose gel after electrophoresis, purified using the NZYGelpure kit (NzyTech, Genes and Enzymes, Lisbon, Portugal) and sequenced in an automated 3130 Genetic Analyser system (Applied Biosystems, Foster City, California, USA) using the BigDye Terminator Cycle sequencing kit (Applied Biosystems). The sequence was submitted to GenBank database and given the accession number MN894670. Using the Basic Local Alignment Search Tool BLASTN (https://blast.ncbi.nlm.nih.gov/Blast.cgi), our sequence revealed high similarity with RHDV2 strains circulating in the Alentejo region in previous years (namely, with strain MG763940).

Parasitological and bacteriological examinations

Parasitological examination revealed light coccidian infections with *Eimeria stidae* in the liver and *E perforans* in the faeces, as well as the intestinal nematodes *Graphidium strigosum* and *Passalurus ambiguous*.

Liver, spleen and lung samples collected from this rabbit cadaver were analysed using standard bacteriological methods. *Staphylococcus xylosus*, a commensal bacterium found in mammals' skin and mucous membranes,[23] was isolated from a pool of organs, including the liver, spleen and lung.

None of these infections explains the animal death.

DISCUSSION

In this study, we report the detection of a juvenile wild rabbit coinfected with RHDV2 and MYXV. This animal was found dead in the South of mainland Portugal in late winter 2019.

Since RHDV, RHDV2 and MYXV have emerged, hundreds of animals were tested worldwide for *Leporipoxvirus* and *Lagovirus*. Interestingly, although both RHD and myxomatosis are mechanically transmitted by arthropod vectors and their dissemination is facilitated by an increase in the wild rabbit population density, there are no previous reports of coinfection. However, interaction between RHD and myxomatosis has been described before.[13–15] Mutze et al[13] proposed, regarding the possible mechanisms of the interaction between RHD and myxomatosis, that when RHD is most active during spring, RHD outcompetes myxomatosis due to the longer incubation time of the latter, which could explain the infrequency of such coinfection events. Assuming similar likelihood of transmission from infective to susceptible animals, RHD would kill most rabbits before they became infective for myxomatosis.[13] In fact, in Australia, the introduction of RHD caused an important change in the timing of myxomatosis outbreaks by displacing these occurrences to autumn and delaying or eliminating the spring–summer outbreaks.[13] Additionally, Marchandeau et al[14] hypothesised, based on serological data, that exposure to one virus could increase the probability of developing the other disease. García-Bocanegra et al[24] showed that seropositivity to lagoviruses was associated with seropositivity to myxomatosis, as 70 per cent of the rabbits that were seropositive to lagoviruses had been also exposed to MYXV.

The coinfection event described here required the interaction between myxomatosis and RHD either by the contemporary overlapping of the two epidemics or a scenario of an endemic situation of myxomatosis plus an epidemic wave of RHDV2.

None of the nine wild rabbit cadavers collected in the same geographical area of the index case between 20 February and 10 May 2019 were simultaneously positive for both viruses, with most of the rabbits testing positive only for RHDV2 (7/9, 77.78 per cent), which suggests an ongoing RHDV2 outbreak.

The occurrence of an RHDV2 outbreak at that time of the year is in accordance with the seasonality of the disease in Iberia. In Portugal, RHDV2 mortality is higher in the cooler months (December–March), in association with the annual inflow of susceptible young rabbits during the breeding season that takes place during the winter and spring, reaching a peak of incidence in January and February.[25] Regarding myxomatosis, the disease can occur throughout the whole year in Europe, although seasonality is observed in Iberia.[24 26 27] As with RHDV2, myxomatosis outbreaks frequently synchronise with the appearance of a large number of susceptible young rabbits, such as the juvenile of this case report, and with the abundance of arthropod vectors.[2] Fleas and mosquitoes are mechanical vectors of MYXV[28 29] and RHDV2,[30–33] and in Portugal, the 2018–2019 winter was mild, favouring to the presence of these arthropods.

Although the clinical history of the juvenile wild rabbit is irretrievable, its poor body condition, compatible with a longer-lasting infection, is more plausible to be caused by MYXV. The severity of the lesions observed in the liver and lungs suggests that RHDV2 infection took place when the animal was already suffering from myxomatosis. The immunosuppressive effect of MYXV increases the risk of other infections,[34] making it possible that infection with MYXV may have favoured RHD virus infection.

In Portugal, viral surveillance of RHD and myxomatosis, both endemic in the entire national territory, was recently strongly intensified within the scope of a national surveillance programme on wild leporids. Since its implementation in August 2017 until August 2019, 1166 wild rabbits legally hunted (n=971) and found dead (n=195) were systematically tested by PCR-based methods for RHDV, RHDV2 and MYXV. For this 2-year period, RHDV2 and MYXV infections were responsible for the death of 70.83 per cent of the animals found in the field, and no coinfections were detected. This may suggest a viral interference phenomenon, in accordance with Mutze et al[13] results. Apart from the coinfected animal, the only wild rabbit from the affected area that tested MYXV positive (1/9, 11.11 per cent) was RHDV2 negative.

Data produced by the ongoing surveillance programme on wild leporids show that the number of positive cases for each disease has been higher in Alentejo and Algarve[35] (NUT 2 regions in the South), compared with the rest of the national territory. In the district of Évora, where the coinfected wild rabbit was collected, the percentages of positivity (PP) in the sample of diseased animals (found dead) for MYXV and RHDV2 were 9.09 per cent (5/55) and 76.37 per cent (42/55), respectively, indicating the contemporary circulation of both viruses in the wild populations. For apparently healthy hunted animals, the PPs found in that district for MYXV and RHDV2 were 6.91 per cent (11/159) and 0 per cent (0/159), respectively.

From August 2017 onwards, apart from the case reported here, no other wild rabbits were found simultaneously infected by both viruses, confirming that this event is extremely rare when compared with single infections by MYXV and RHDV2. In the group of diseased wild rabbits found dead in the field, sampled between August 2017 and August 2019, coinfection represents 0.52 per cent (1/195), much lower than single infections by MYXV (14.35 per cent, 28/195) and RHDV2 (55.38 per cent, 108/195). Our results reflect the high pathogenicity of both viruses, showing that more than half of the animals found in the field died of RHDV2 infection, while MYXV accounted for one in seven of the deaths.

As expected, the PPs for the group of hunted animals are much lower for both viruses, namely 4.33 per cent (42/971) for MYXV and 0.62 per cent (6/971) for RHDV2. The differences in the PP ratios (found dead/hunted), 89.9 per cent for RHDV2 and 3.3 per cent for MYXV, indicate a shorter course disease for RHDV2 infections and higher mortality compared with myxomatosis.

To our knowledge, viral coinfections were never reported in wild rabbits, despite in other species, this event is common. Examples of coinfections are provided by MYXV and leporid herpesvirus 5 in the Iberian hare,[36] feline herpesvirus 1 and feline calicivirus in cats,[37] or peste des petits ruminants virus and foot-and-mouth disease virus in goats.[38]

Generally, coinfections are believed to exert a negative effect on the hosts' health, but little is yet known about the effect that one pathogen has on the other and on the implications of coinfection to the host. Viral interference, where one virus competitively suppresses replication of the other coinfecting viruses, is the most common outcome of coinfection. It may be mediated by various factors, such as interferons, defective interfering particles and cellular factors, among others. Nevertheless, coinfections of certain viruses may also promote an increase or may have no effect on virus replication, allowing, in the latter, for coinfecting viruses to coexist (accommodation), (Reviewed in Kumar et al).[39]

Although from an epidemiological point of view the impact of the coinfection by RHDV2 and MYXV in the wild rabbit populations appears minor since it is a rare event, this possibility must be taken into account, especially in the context of diagnosis, where it is common to attribute a viral disease to the infection by a single agent. The contribution of multiple-agent infections in the clinical outcome is rarely considered, which may lead to failure in the detection of additional agents.[39] In the case reported here, the molecular diagnosis for MYXV and RHDV2 were also corroborated by the observation of histopathological lesions typical of both diseases.

More than reporting an RHDV2–MYXV coinfection event in a wild rabbit, this paper aimed to raise awareness of the need to perform both diagnoses in areas where the two diseases are endemic, even when skin lesions are not yet present.

Learning points

- ► During a 2-year period, starting in August 2017, a national ongoing surveillance programme on wild leporids tested 1166 wild rabbits for myxoma virus (MYXV) and rabbit haemorrhagic disease virus 2 (RHDV2).
- ► In the sampling group diseased animals found dead, 14.35 per cent (28/195) and 55.38 per cent (108/195) were found positive for MYXV and RHDV2, respectively.
- ► A coinfection with MYXV and RHDV2 was detected in one juvenile wild rabbit found dead in Alentejo, Évora district, South Portugal.
- ► MYXV and RHDV2 coinfection is a rare event compared with single infections, representing a relative frequency of 0.52 per cent (1/195) in the group of diseased animals found dead.

experiments and wrote and revised the manuscript. All authors discussed the results and contributed critically to the final document.

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REFERENCES

1 Delibes-Mateos M, Ferreira C, Carro F, *et al*. Ecosystem effects of variant rabbit hemorrhagic disease virus, Iberian Peninsula. *Emerg Infect Dis* 2014;20:2166–8.
2 Villafuerte R, Castro F, Ramírez E, *et al*. Large-scale assessment of myxomatosis prevalence in European wild rabbits (*Oryctolagus cuniculus*) 60years after first outbreak in Spain. *Res Vet Sci* 2017;114:281–6.
3 Murphy FA, Fauquet CM, Bishop DHL, *et al*. - virus taxonomy: classification and nomenclature of viruses. sixth report of the International Committee for the taxonomy of viruses. Archives of virology, 1995. Vienna: Springer Verlag: 586.
4 Fenner F, Ratcliffe FN. *Myxomatosis*. Cambridge University Press, 1965.
5 Guixeras J. *Tratado de la mixomatosis*. Graf. Bat, Salt, Girona, 1957: 126 pp.
6 Le Gall-Reculé G, Zwingelstein F, Boucher S, *et al*. Detection of a new variant of rabbit haemorrhagic disease virus in France. *Vet Rec* 2011;168:137–8.
7 Le Gall-Reculé G, Lavazza A, Marchandeau S, *et al*. Emergence of a new lagovirus related to rabbit haemorrhagic disease virus. *Vet Res* 2013;44:81.
8 Lopes A, Correia J, Abrantes J, *et al*. Is the new variant RHDV replacing genogroup 1 in Portuguese wild rabbit populations? *Viruses* 2015;7:27–36.
9 Mahar JE, Hall RN, Peacock D, *et al*. Rabbit hemorrhagic disease virus 2 (RHDV2; GI.2) is replacing endemic strains of RHDV in the Australian landscape within 18 months of its arrival. *J Virol* 2018;92:e01374–17.
10 Liu SJ, Xue HP, BQ P, *et al*. A new viral disease of rabbits. *Animal Husbandry Vet Med* 1984;16:253–5.
11 Rouco C, Aguayo-Adán JA, Santoro S, *et al*. Worldwide rapid spread of the novel rabbit haemorrhagic disease virus (GI.2/RHDV2/b). *Transbound Emerg Dis* 2019;66:1762–4.
12 Abrantes J, van der Loo W, Le Pendu J, *et al*. Rabbit haemorrhagic disease (RhD) and rabbit haemorrhagic disease virus (RHDV): a review. *Vet Res* 2012;43:12.
13 Mutze G, Bird P, Kovaliski J, *et al*. Emerging epidemiological patterns in rabbit haemorrhagic disease, its interaction with myxomatosis, and their effects on rabbit populations in South Australia. *Wildlife Research* 2002;29:577–90.
14 Marchandeau S, Bertagnoli S, Peralta B, *et al*. Possible interaction between myxomatosis and calicivirosis related to rabbit haemorrhagic disease affecting the European rabbit. *Vet Rec* 2004;155:589–92.
15 García-Bocanegra I, Astorga RJ, Napp S, *et al*. Factors affecting the seroprevalence of lagovirus infection in wild rabbits (*Oryctolagus cuniculus*) in southern Spain. *Vet J* 2011;189:89–94.
16 Fulford GR, Lee XJ, Berman D, *et al*. Interaction of myxomatosis and rabbit haemorrhagic disease in wild rabbit. 19th international Congress on modelling and simulation, Perth, Australia, 2011.
17 Cook HC. Origins of … tinctorial methods in histology. *J Clin Pathol* 1997;50:716–20.
18 Duarte MD, Carvalho CL, Barros SC, *et al*. A real time TaqMan RT-PCR for the detection of rabbit hemorrhagic disease virus 2 (RHDV2). *J Virol Methods* 2015;219:90–5.
19 Duarte MD, Barros SC, Henriques AM, *et al*. Development and validation of a real time PCR for the detection of myxoma virus based on the diploid gene M000.5L/R. *J Virol Methods* 2014;196:219–24.
20 Tham KM, Barnes SM, Hunter SN. Polymerase chain reaction amplification and gene sequence analysis of a calicivirus from a feral rabbit. *Virus Genes* 1999;18:235–42.
21 Le Gall G, Arnauld C, Boilletot E, *et al*. Molecular epidemiology of rabbit haemorrhagic disease virus outbreaks in France during 1988 to 1995. *J Gen Virol* 1998;79:11–16.
22 Duarte M, Carvalho C, Bernardo S, *et al*. Rabbit haemorrhagic disease virus 2 (RHDV2) outbreak in Azores: disclosure of common genetic markers and phylogenetic segregation within the European strains. *Infect Genet Evol* 2015;35:163–71.
23 Dordet-Frisoni E, Dorchies G, De Araujo C, *et al*. Genomic diversity in *Staphylococcus xylosus*. *Appl Environ Microbiol* 2007;73:7199–209.
24 García-Bocanegra I, Astorga RJ, Napp S, *et al*. Myxomatosis in wild rabbit: design of control programs in Mediterranean ecosystems. *Prev Vet Med* 2010;93:42–50.
25 Rouco C, Abrantes J, Serronha A, *et al*. Epidemiology of RHDV2 (Lagovirus europaeus/GI.2) in free-living wild European rabbits in Portugal. *Transbound Emerg Dis* 2018;65:e373–82.
26 Ross J, Tittensor AM. The establishment and spread of myxomatosis and its effect on rabbit populations. *Philos Trans R Soc Lond B Biol Sci* 1986;314:599–606.
27 Simón MC, Ortega C, Maynar P, *et al*. Studies in wild rabbit (Oryctolagus cuniculus) populations in Navarra (Spain). II. epidemiology of myxomatosis. *Gib Faun Sauv Gam* 1998;15:103–21.
28 Fenner F, Day MF, Woodroofe GM. Epidemiological consequences of the mechanical transmission of myxomatosis by mosquitoes. *J Hyg* 1956;54:284–303.
29 Flowerdew JR, Trout RC, Ross J. Myxomatosis: population dynamics of rabbits (Oryctolagus cuniculus Linnaeus, 1758) and ecological effects in the United Kingdom. *Rev Sci Tech* 1992;11:1109–13.
30 Lenghaus C, Westbury H, Collins B, *et al*. Overview of the RHD project in Australia. In: *Rabbit haemorrhagic disease: issues for assessment for biological control (R.K. Munro and R.T. Williams*. Canberra: Australian Government Printing Service, 1994: 104–29.
31 Asgari S, Hardy JR, Sinclair RG, *et al*. Field evidence for mechanical transmission of rabbit haemorrhagic disease virus (RHDV) by flies (Diptera:Calliphoridae) among wild rabbits in Australia. *Virus Res* 1998;54:123–32.
32 Cooke BD. Rabbit haemorrhagic disease: field epidemiology and the management of wild rabbit populations. *Rev Sci Tech* 2002;21:347–58.
33 McColl KA, Merchant JC, Hardy J, *et al*. Evidence for insect transmission of rabbit haemorrhagic disease virus. *Epidemiol Infect* 2002;129:655–63.
34 Marlier D, Mainil J, Linde A, *et al*. Infectious agents associated with rabbit pneumonia: isolation of amyxomatous myxoma virus strains. *Vet J* 2000;159:171–8.
35 Duarte M, Carvalho CL, Santos FA, *et al*. "+Coelho: Avaliação Ecossanitária das Populações Naturais de Coelho Bravo Visando o Controlo da Doença Hemorrágica Viral". Relatório de atividades na época venatória 2017-2018. INIAV I.P., DGAV, CIBIO, ANPC, CNCP, FENCAÇA. Fundo Florestal Permanente. ICNF., 2018.
36 Abade dos Santos FA, Monteiro M, Pinto A, *et al*. First description of a herpesvirus infection in genus *Lepus*. BioRxiv. 2020 https://doi.org/
37 Zicola A, Saegerman C, Quatpers D, *et al*. Feline herpesvirus 1 and feline calicivirus infections in a heterogeneous cat population of a rescue shelter. *J Feline Med Surg* 2009;11:1023–7.
38 Kumar N, Barua S, Riyesh T, *et al*. Complexities in isolation and purification of multiple viruses from mixed viral infections: viral interference, persistence and exclusion. *PLoS One* 2016;11:e0156110.
39 Kumar N, Sharma S, Barua S, *et al*. Virological and immunological outcomes of coinfections. *Clin Microbiol Rev* 2018;31:e00111–7.

Chapter 4

Research papers on Myxoma virus and Herpesvirus

■■

Chapter 4 includes several international scientific publications of research on myxoma virus of Iberian hare and wild rabbit and herpesvirus.

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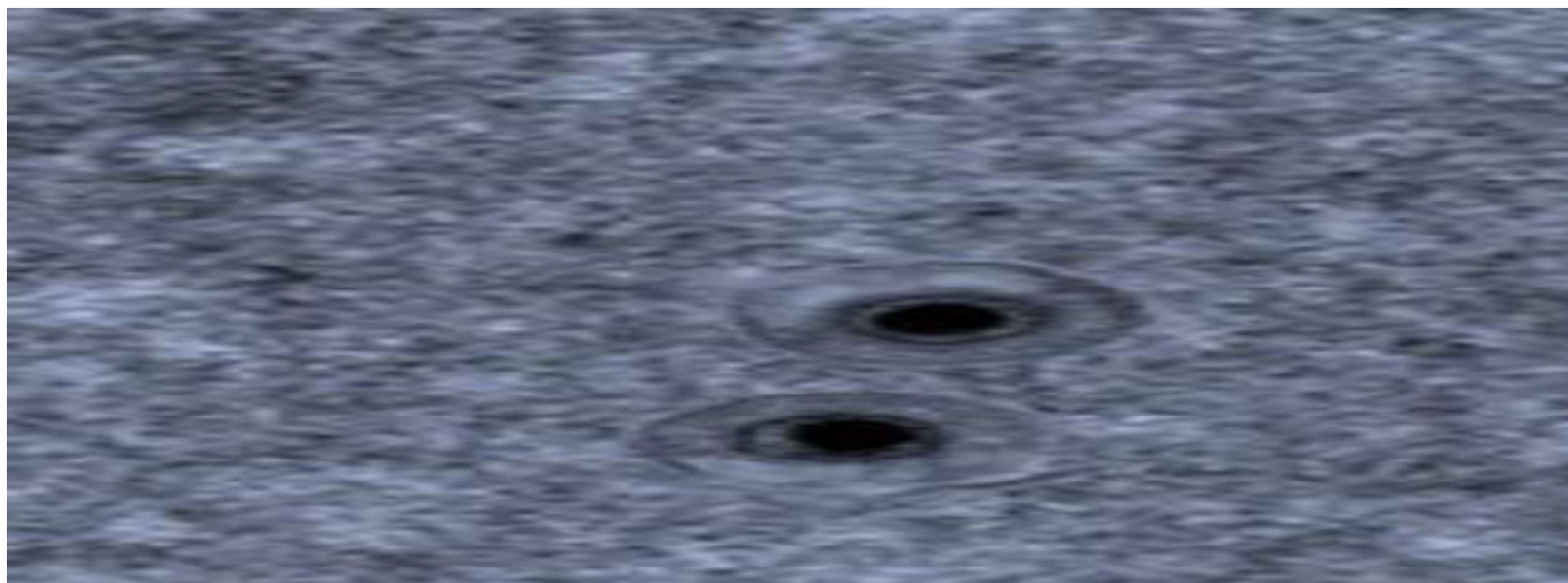
Which myxoma virus strains caused the outbreak of myxomatosis in Iberian hare in Portugal?

Scientific publication 9

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

■ ■

Myxomatosis affects rabbit species since 1896 and European hare species, but not the Iberian hare. In autumn 2018, a natural recombinant myxoma virus emerged in Portugal in Iberian hares, after being detected a few months before in Spain. Like in Spain, in Portugal the ability to infect hares was linked to the insertion of an additional 2.8 Kpb long piece of the genome.

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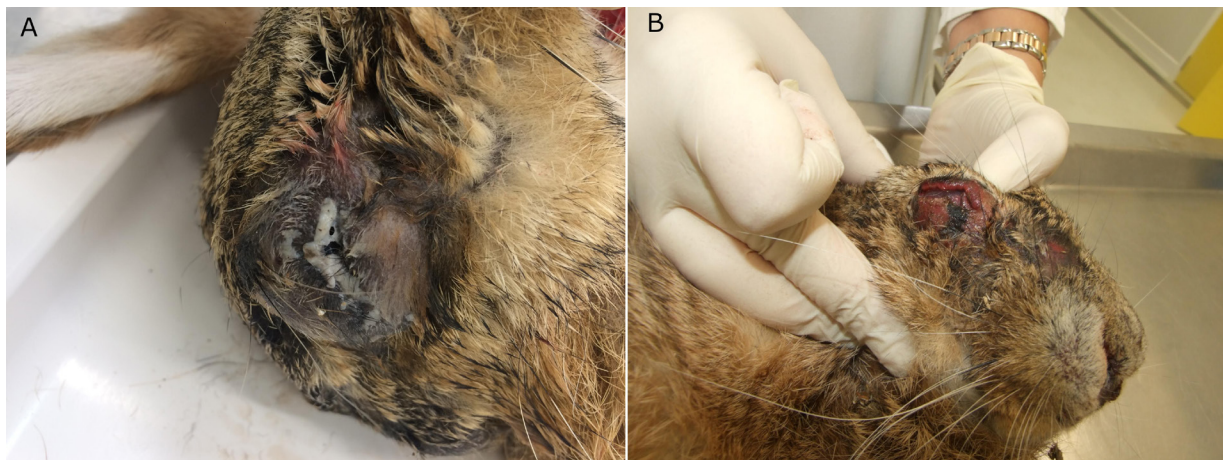
Figure 1 Macroscopic lesions found in the Iberian hares. Oedema of the eyelids and conjunctival mucopurulent discharge (A) observed in hare 1. Eyelid thickening with small diffuse nodular lesions (B) found in hare 2.

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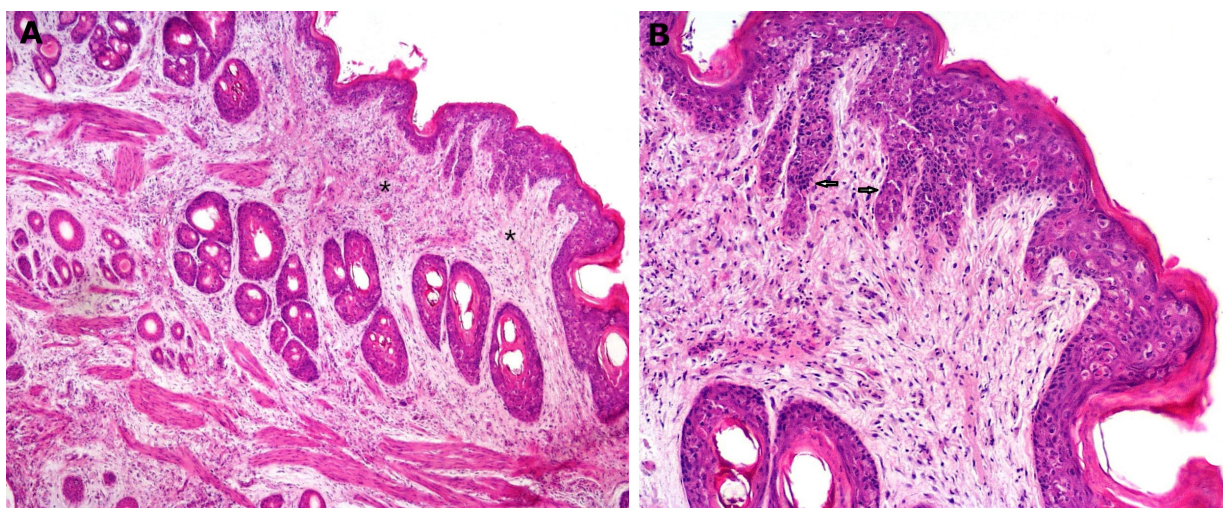
Figure 2 Histopathological features of the eyelid lesions observed in hare 1. (A) Abundant myxoid tissue indicated by black stars (x 40 magnification) and (B) hyperplasia of the epidermis pointed by arrows and the presence of myxoid tissue in the dermis (x 100 magnification). H&E staining.

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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REFERENCES

1 Murphy FA, Fauquet CM, Bishop DHL, *et al*. Virus taxonomy: the database of the International Committee on Taxonomy of Viruses (ICTV). *Arch. Virol* 1995;ement 10:586.

2 Sanarelli G. Virus Dmyxomatogene. Beitrag zum stadium Der Krankheit-serreger ausserhalbdes Sichtbaren. *Zbl Bakt* 1898;23:865–73.

3 Kerr PJ, Best SM. Myxoma virus in rabbits. *Rev Sci Tech* 1998;17:256–68.

4 Stanford MM, Werden SJ, McFadden G. Myxoma virus in the European rabbit: interactions between the virus and its susceptible host. *Vet Res* 2007;38:299–318.

5 Kerr PJ, Liu J, Cattadori I, *et al*. Myxoma virus and the Leporipoxviruses: an evolutionary paradigm. *Viruses* 2015;7:1020–61.

6 Fenner F. Adventures with poxviruses of vertebrates. *FEMS Microbiol Rev* 2000;24:123–33.

7 Marlier D, Vindevogel H. Poxless myxomatosis—isolation of three strains in Belgium. *Ann Med Vet* 1996;140:343–6.

8 Marlier D, Mainil J, Linde A, *et al*. Infectious agents associated with rabbit pneumonia: isolation of amyxomatous myxoma virus strains. *Vet J* 2000a;159:171–8.

9 Marlier D, Mainil J, Sulon J, *et al*. Study of the virulence of five strains of amyxomatous myxoma virus in crossbred New Zealand White/Californian conventional rabbits, with evidence of long-term testicular infection in recovered animals. *J Comp Pathol* 2000b;122:101–13.

10 Fenner F, Ratcliffe FN. *Myxomatosis*. Cambridge University Press, 1965.

11 Kerr PJ, Ghedin E, DePasse JV, *et al*. Evolutionary history and attenuation of myxoma virus on two continents. *PLoS Pathog* 2012;8:8.

12 Fenner F, Marshall ID. A comparison of the virulence for European rabbits (*Oryctolagus cuniculus*) of strains of myxoma virus recovered in the field in Australia, Europe and America. *J Hyg* 1957;55:149–91.

13 Marshall ID, Fenner F. Studies in the epidemiology of infectious myxomatosis of rabbits. V. Changes in the innate resistance of Australian Wild rabbits exposed to myxomatosis. *J Hyg* 1958;56:288–302.

14 Best SM, Kerr PJ. Coevolution of host and virus: the pathogenesis of virulent and attenuated strains of myxoma virus in resistant and susceptible European rabbits. *Virology* 2000;267:36–48.

15 Barlow A, Lawrence K, Everest D, *et al*. Confirmation of myxomatosis in a European brown hare in Great Britain. *Vet Rec* 2014;175:75–6.

16 Saari SAM, Rudbäck E, Niskanen M, *et al*. Contagious mucocutaneous dermatitis of the mountain hare (*Lepus timidus*): pathology and cause. *J Wildl Dis* 2005;41:775–82.

17 Lucas A, Bouley G, Quinchon C, *et al*. La myxomatose Du lievre. *Bulletin de l'Office International des Epizooties* 1953;39:770–6.

18 García-Bocanegra I, Camacho-Sillero L, Risalde MA, *et al*. First outbreak of myxomatosis in Iberian hares (*Lepus granatensis*). *Transbound Emerg Dis* 2019;66:2204–8.

19 Águeda-Pinto A, Lemos de Matos A, Abrantes M, *et al*. Genetic characterization of a recombinant myxoma virus in the Iberian hare (*Lepus granatensis*). *Viruses* 2019;11:pii: E530.

20 Dalton KP, Martín JM, Nicieza I, *et al*. Myxoma virus jumps species to the Iberian hare. *Transbound Emerg Dis* 2019;66:2218–26.

21 Cook HC. Origins of tinctorial methods in histology. *J Clin Pathol* 1997;50:716–20.

22 Duarte MD, Barros SC, Henriques AM, *et al*. Development and validation of a real time PCR for the detection of myxoma virus based on the diploid gene M000.5L/R. *J Virol Methods* 2014;196:219–24.

23 OIE. Myxomatosis, Portugal, 2018. Available: https://www.oie.int/wahis_2/public/wahid.php/Reviewreport/Review?page_refer=MapFullEventReport&reportid=28628

24 Tham KM, Barnes SM, Hunter SN. Polymerase chain reaction amplification and gene sequence analysis of a calicivirus from a feral rabbit. *Virus Genes* 1999;18:235–42.

25 Duarte MD, Carvalho CL, Barros SC, *et al*. A real time TaqMan RT-PCR for the detection of rabbit hemorrhagic disease virus 2 (RHDV2). *J Virol Methods* 2015;219:90–5.

26 Gortazar C, Millán J, Acevedo P, *et al*. A large-scale survey of brown hare *Lepus europaeus* and Iberian hare *L. granatensis* populations at the limit of their ranges. *Wildlife Biol* 2007;13:244–50.

27 Soriguer R, Carro F. *Lepus granatensis*. The IUCN Red List of Threatened Species 2019: e.T41306A2953195, 2019. Available: http://dx.doi.org/10.2305/IUCN.UK.2019-1.RLTS.T41306A2953195.en [Accessed 02 Dec 2019].

28 Lopes de Carvalho I, Toledo A, Carvalho CL, *et al*. *Francisella* species in ticks and animals, Iberian Peninsula. *Ticks Tick Borne Dis* 2016;7:159–65.

29 Duarte J, ibérica L. *Lepus granatensis* (Rosenhauer, 1856). *Galemys* 2000;12:3–14.

30 Purroy FJ. Liebre ibérica—*Lepus granatensis*. En: Enciclopedia Virtual de los Vertebrados Españoles. In: Salvador A, Cassinello J, eds. *Museo Nacional de Ciencias Naturales, Madrid*, 2011. http://www.vertebradosibericos.org/

31 Duarte M, Carvalho CL, Santos FA, *et al*. +Coelho: Avaliação Ecossanitária das Populações Naturais de Coelho Bravo Visando o Controlo da Doença Hemorrágica Viral". Relatório de atividades na época venatória 2017-2018. INIAV I.P., DGAV, CIBIO, ANPC, CNCP, FENCAÇA. Fundo Florestal Permanente. ICNF 2018.

32 Acevedo P, Melo-Ferreira J, Real R, *et al*. Past, present and future distributions of an Iberian endemic, Lepus granatensis: ecological and evolutionary clues from species distribution models. *PLoS One* 2012;7:e51529.

33 Ministerio de Agricultura, Pesca Y Alimentación: situación de brote de mixomatosis en liebre ibérica. Available: https://www.mapa.gob.es/es/ganaderia/temas/sanidad-animal-higiene-ganadera/notamixomatosis07112019_tcm30-482844.pdf [Accessed 7 Nov 2019].

34 Fenner F, Day MF, Woodroofe GM. Epidemiological consequences of the mechanical transmission of myxomatosis by mosquitoes. *J Hyg* 1956;54:284–303.

Scientific publications

10 Detection of Recombinant Hare Myxoma Virus in Wild Rabbits (*Oryctolagus cuniculus algirus*)

and

11 Recombinant myxoma virus infection associated with high mortality in rabbit farming (*Oryctolagus cuniculus*)

■ ■

The natural recombinant myxoma virus (ha-MYXV) was not detected in rabbits following its detection in hares, suggesting a species-specific tropism. However, in 2020, ha-MYXV was detected in domestic and wild rabbits demonstrating its ability to infect rabbits.

Article

Detection of Recombinant Hare Myxoma Virus in Wild Rabbits (*Oryctolagus cuniculus algirus*)

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Abstract: In late 2018, an epidemic myxomatosis outbreak emerged on the Iberian Peninsula leading to high mortality in Iberian hare populations. A recombinant Myxoma virus (strains MYXV-Tol and ha-MYXV) was rapidly identified, harbouring a 2.8 kbp insertion containing evolved duplicates of M060L, M061L, M064L, and M065L genes from myxoma virus (MYXV) or other Poxviruses. Since 2017, 1616 rabbits and 125 hares were tested by a qPCR directed to M000.5L/R gene, conserved in MYXV and MYXV-Tol/ha-MYXV strains. A subset of the positive samples (20%) from both species was tested for the insert with MYXV being detected in rabbits and the recombinant MYXV in hares. Recently, three wild rabbits were found dead South of mainland Portugal, showing skin oedema and pulmonary lesions that tested positive for the 2.8 kbp insert. Sequencing analysis showed 100% similarity with the insert sequences described in Iberian hares from Spain. Viral particles were observed in the lungs and eyelids of rabbits by electron microscopy, and isolation in RK13 cells attested virus infectivity. Despite that the analysis of complete genomes may predict the recombinant MYXV strains' ability to infect rabbit, routine analyses showed species segregation for the circulation of MYXV and recombinant MYXV in wild rabbit and in Iberian hares, respectively. This study demonstrates, however, that recombinant MYXV can effectively infect and cause myxomatosis in wild rabbits and domestic rabbits, raising serious concerns for the future of the Iberian wild leporids while emphasises the need for the continuous monitoring of MYXV and recombinant MYXV in both species.

Keywords: myxomatosis; recombinant myxoma virus; ha-MYXV; European rabbit; *Oryctolagus cuniculus algirus*; species jump; spillover

1. Introduction

In the Mediterranean ecosystems, wild rabbit is an important prey for more than 40 predatory aerial and terrestrial species, some of which are endangered [1]. It also plays a crucial role as a soil "architect", contributing to seed dispersal, and landscaping [2]. Besides its ecological role, the wild rabbit is an important game species economically and socially.

Myxoma virus (MYXV) and rabbit haemorrhagic disease virus 2 (RHDV2) are the two major pathogen threats for the European rabbit (*Oryctolagus cuniculus*), and may occasionally be found simultaneously [3]. The etiological agent of myxomatosis is MYXV, a double-stranded DNA *Leporipoxvirus* of the family Poxviridae [4].

Myxomatosis is an endemic disease of South American rabbits and was first described in laboratory rabbits in 1898 in Uruguay [5]. The disease is characterised by the presence of nodules in the skin surrounding the eyes, nose, mouth, ears, and genitalia. Conjunctivitis, accompanied by purulent discharge is frequently found as a signal of disease [6].

Despite these signs being the most commonly found in the classic, nodular or typical form of disease, myxomatosis can also be found as a respiratory form (amyxomatous form), with variable degrees of severity, where cutaneous signals are minor or not observed [7–9]. The origin of this amyxomatous virus is still unclear. Viral mutations and reactivation of subclinical infections are two of the hypotheses proposed [10].

Regardless, the two disease presentations, myxomatosis was considered a rabbit disease during many decades, with some scarce reports in European hares [11,12].

Accordingly, during a National Leporid Surveillance Program (Project +Coelho, Dispatch 4757/17, 31th may), that started in mid-2017, 92 hares and 903 rabbits, collected until October 2018, were analysed for MYXV-DNA using a qPCR directed to the diploid gene M000.5L/R, which is conserved in MYXV and recombinant MYXV strains. Until this date, no hare was positive for any MYXV strain.

The emergence of myxomatosis in the Iberian hare in mid 2018, was caused by a recombinant myxoma virus (first designated as MYXV-Tol, and subsequently ha-MYXV considering its modified tropism towards hares), harbouring an insertion of about 2.8 kbp [13–16]. After this, health surveillance in the Iberian hare within the scope of Project +Coelho (investigating MYXV [17], RHDV2 [18] and LeHV-5 [19]) and in the wild rabbit (investigating MYXV and RHDV2 [18]) was extended to include screening for ha-MYXV as described by Dalton et al. [15].

The detection of a recombinant MYXV in hares, and the apparent segregated circulation of classical MYXV in rabbits and ha-MYXV in hares, initially suggested the adaptation of MYXV to hares in order to efficiently multiply in this species. Given that hare MYXV, originally considered hare specific, is also being detected in rabbits, who succumbed to the disease, a more generalist designation, geographic and species independent, such as rec-MYXV (for recombinant myxoma virus), may be preferable for the future.

Until the cases reported here, in all tested samples, classic MYXV was only found in wild rabbits and recombinant MYXV in Iberian hares. To our knowledge, we are reporting for the first time, the detection of myxomatosis in European rabbit caused by the recombinant MYXV, adding concerns to the already fragile conservation state of the wild rabbit, taking into account its threat of extinction [20].

2. Materials and Methods

2.1. Sample, Necropsy and Histopathology

Two adult wild rabbits (*Oryctolagus cuniculus algirus*), in good body condition, found dead in June 2020 (Male, 15758PT20) and July 2020 (female, 20545PT20, from here named Female 1) in the same hunting reserve in Moura, district of Beja, and one wild rabbit in good body condition, found dead in August 2020 in Samora Correia, district of Santarém (female, 22660PT20, from here named Female 2) were collected and investigated within the scope of a national surveillance program in action since August 2017.

Necropsy was performed according to routine procedures, and samples were collected for bacteriology (liver, spleen and lung), parasitology (gastrointestinal tract and liver), histopathology (lung, liver, spleen, kidney, eyelid and genitalia) and virology (liver, spleen, lung, kidney, eyelid andgenitalia).

For histopathology, skin and genitalia fragments were fixated in 10% neutral buffered formalin (*w/v*), routinely paraffin embedded, sectioned at 4 μm, and stained with Hematoxylin and Eosin (H&E).

2.2. Parasitological and Bacteriological Examination

Parasitological examination of the intestine was carried out resourcing to direct wet mount, sedimentation and filtration techniques. Liver, spleen and lung samples were analysed using standard bacteriological methods. Enterobacteriaceae and non-Enterobacteriaceae were tested using the ID 32E (Biomerieux®, Lisbon, Portugal) test and the API 20NE kit (Biomerieux®) test respectively. The presence of Streptococcus and Staphylococcus was investigates using the ID 32 STREPT (Biomerieux®) and the ID 32 STAPH kits (Biomerieux®), respectively. The API CORYNE (Biomerieux®) kit was used for the identification of Corynebacteria and coryne-like organisms. For Salmonella, peptone water and Rappaport Vassiliadis semi solid culture media were used. Whenever there was a suspicion of Salmonella, the agarose SMID2 and XLD culture media were used. Other culture media for bacterial identification in the samples included the MacConkey agar and the Blood agar culture media.

2.3. Virological and Serological Examinations

For nucleic acid extraction, fresh samples of liver and spleen, kidney, lung, eyelid and genitalia were homogenised at 20% (*w/v*) with phosphate buffered saline (PBS) and clarified at 3000 *g* for 5 min. Total DNA and RNA were extracted from 200 μL of the clarified supernatants, using the MagAttract 96 cador Pathogen Kit (Qiagen, Hilden, Germany) in a BioSprint 96 nucleic acid extractor (Qiagen, Hilden, Germany), according to the manufacturer's protocol.

The rabbits were tested for rabbit haemorrhagic disease virus 2 (RHDV2) and MYXV by real time PCR targeting the M000.5 L/R gene [17,18]. The 2.8 kbp insert was investigated by the system described by Dalton et al. [15] using primers 9A/9B and 9E/9F that flank the insertion, allowing the amplification of a 3.1 or 4.6 kbp region in recombinant MYXV or a 300 bp region (absence of insert, using the oligomers 9E/9F) in MYXV. Amplification reactions were carried out in a Bio-Rad CFX96™ Thermal Cycler (Bio-Rad Laboratories Srl, Redmond, USA), using the One Step RT-PCR kit (Qiagen, Hilden, Germany) for RHDV2, and the HighFidelity PCR Master Mix (Roche Diagnostics GmbH, Mannheim, Germany), for MYXV and recombinant MYXV.

A commercial kit (Civtest® Cuni Mixomatosis—Hipra, Girona, Spain) developed for the detection of rabbit MYXV antibodies was validated for hare sera [21] and used to detect MYXV antibodies, following the manufacturer's instructions. For Female 1, serosanguinolent thoracic fluid was used instead of serum due to blood coagulation.

2.4. Sequencing Analysis

The initial PCR products (≈3100 bp or ≈4600 bp) encompassing the 2.8 kbp insert, were visualised in 2% horizontal electrophoresis agarose gel, purified using the NZYGelpure kit (Nzytech, Lisbon, Portugal), and directly sequenced using the ABI Prism BigDye Terminator v3.1 Cycle sequencing kit (Thermo Fisher Scientific, Waltham, MA, USA) on a 3130 Genetic Analyser (Applied Biosystems, Foster City, CA, USA). Sequencing by primer walking was carried out, with a total of 12 additional oligomers designed for the purpose (Table 1).

The two nucleotide sequences obtained (15758PT20 and 20545PT20) were assembled using the Seqscape Software v2.7 (Applied Biosystems, Foster City, CA, USA), and submitted to GenBank (MT940239 and MT940240).

2.5. Isolation

Isolation of MYXV from the rabbits' tissues (15758PT20, 20545PT20 and 22660PT20) was achieved separately from eyelid, genitalia and lung. Samples were homogenised at 20% (*w/v*) in PBS containing penicillin, streptomycin and amphotericin B (antibiotic-antimycotic), used according to

the manufacturer (Gibco,Massachusetts, EUA). Following centrifugation (3000 *g*, 10 min), the supernatant was filtered through a 0.45-μm-pore-size filter (Millipore Express, Darmstad, Germany) and used to inoculate sub confluent (70%) Rabbit Kidney (RK13) cells (ATCC-CCL-37). RK13 cells were grown in Eagle′s medium supplemented with 5% foetal calf serum (Gibco), penicillin, streptomycin and amphotericin B (antibiotic-antimycotic used at 1:100, Gibco) and 50 μg/mL gentamicin (Gibco). Cells were maintained at 37 °C under humidified atmosphere with 5% CO_2 and observed daily for cytopathic effect (CPE) by phase contrast microscopy. The supernatant and cell pellet of each passage were tested for the presence of MYXV by qPCR [17].

The isolation of the virus allowed verifying its viability in the rabbit tissues, inferred by the cytopathic effect and in-house immunofluorescence protocol using MYXV positive hare serum (*protocol available on request*). The photographs were taken using an Inverted research microscope, Nikon Eclipse Ti (Nikon Instruments Europe, Amsterdam, Netherlands).

Table 1. Summary of oligomers used for amplification and sequencing.

Primer Name	Sequence (5′-3′)	Position in MK340973 (nt)	Reference
9B (forward)	CGCAGGTCCACGTATAAACC	11458-11477 and 153103-153084	[15]
9A (reverse)	CGAACGTATCATTAGACAATG	16060-16040	
9E (forward)	CTTCGTCTACGCCTCCTACG	12116-12135	
9F (reverse)	GCGTCGTTGTGGTCAGACAGAG	15256-15243	
305R (reverse)	AACCCGCACAACGTAAAGTACC	12420-12399	This manuscript
448F (forward)	GTCATATTCCTGATTTGGGTAATC	12563-12587	
796R (reverse)	AGGAGGAAAAGAACCTATGACAC	12911-12889	
1003F (forward)	GTGTGTACCTGGTGCAGAACC	13118-13138	
1302R (reverse)	TGAAGATGATAATGATGATGAATATCG	13417-13391	
1467F (forward)	TTCATCGTTTATGGGAAAATCTATG	13582-13606	
1819R (reverse)	GAGGGGACAGTTATGGATGTAC	13934-13913	
2028F (forward)	AAGATGCGTCTGTGTAACAATCC	14143-14165	
2325R (reverse)	AACAATGTATACACTCATGACAGTAC	14440-14415	
2458F (forward)	ATGGCCATCGTAAGTTGCCATG	14573-14594	
2847R (reverse)	CAGAGTACTTAGATTTTCTGCTAG	14962-14939	
2954F (forward)	ATCCATTGTTCGTCAGTAGATCG	15069-15091	

2.6. Electron Microscopy

The fragments selected (eyelid and lung) for transmission electron microscopy (TEM) were placed in 10% buffered formalin (*w/v*). Samples were then washed and transferred to 0.05M cacodylate buffer containing 2.5% glutaraldehyde, and post-fixed with aqueous 1% osmium tetroxide (EMS) for 1 hour, fragments were then stained in block with ready-to-use UA-zero (Agar Scientifics, Essex, United Kingdom), after which they were dehydrated in increasing concentrations of ethanol, infiltrated and embedded in Araldite resin (Agar Scientifics). Polymerisation was performed at 60 °C for 2 days. Ultrathin sections were cut using a Reichert ultracut E ultramicrotome (Leica, Wetzlar, Germany), collected to 1% 200 mesh copper grids (Agar Scientifics), and examined in a Jeol 1400plus transmission electron microscope at an accelerating voltage of 120 kV. Digital images were obtained using an AMT XR16 bottom mount digital camera (AMT©, Woburn, MA, USA). The sections were systematically analysed using AMT© software and several high and low magnifications were acquired.

3. Results

3.1. Necropsy and Histopathology

The Male wild rabbit had mild swelling of the eyelids (Figure 1), Female 1 mild swelling of the eyelids and vulva (Figure 2) and Female 2 nodular thickening of the right ear and erosive lesions in the muzzle. Histopathology of the lungs showed focal alveolar oedema with hyaline substance deposits in the alveolar septa in the Male (Figure 3) and infiltration of alveolar septa by mononucleated cells and focal necrosis of alveolar septa with deposits of hyaline substance in Female

1. There was bacterial infiltration in the lung parenchyma of the Male rabbit. The eyelid of the Male presented oedema with epidermal detachment (Figure 4). Due to autolysis, the histopathologic analysis of Female 2 was impaired.

Figure 1. Mild oedema of the eyelid and presence of serous discharge (Male).

Figure 2. Oedema of the vulva (Female 1)**.**
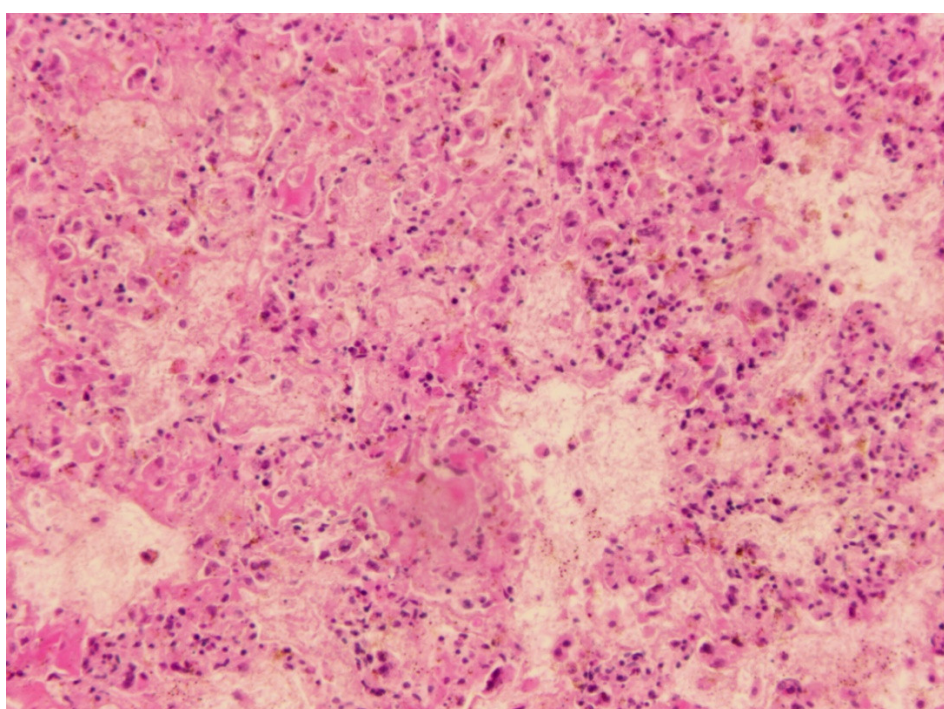
Figure 3. Microscopic finding in Female 1. Infiltration of alveolar septa by mononucleated cells and focal necrosis of alveolar septa with deposits of hyaline substance (H&E, 100×).
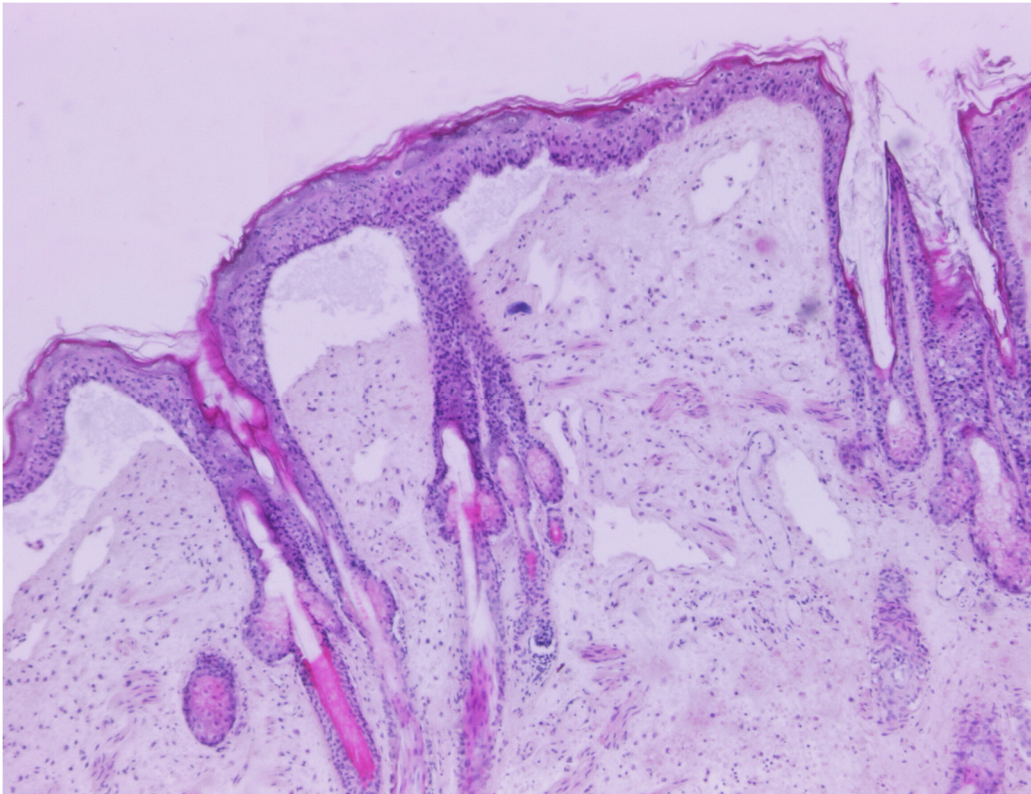
Figure 4. Microscopic finding in Male. Eyelid presenting oedema with epidermal detachment (H&E, 40×).

3.2. Virological, Bacteriological and Parasitological Results

The three animals tested negative for RHDV, RHDV2 and LeHV-5. The Cq values obtained with the qPCR targeting the diploid MYXV gene M000.5L/R in the tissues from both rabbits revealed high viral charges in the liver/spleen (Male = 2.01×10^9 copies/mg; Female 1 = 1.88×10^9 copies/mg; Female 2 = 1.83×10^{10}), lung (Male = 1.3×10^{10} copies/mg; Female = 1.53×10^9 copies/mg; Female 2 = 2.31×10^9), eyelid (Male = 1.13×10^{10} copies/mg; Female 1 = 2.41×10^{10} copies/mg; Female 2 = 1.25×10^{11}), genitalia (Male = 7.47×10^9 copies/mg, Female 1 = 1.30×10^{10} copies/mg; Female 2 = 3.05×10^9) and kidney (Male = not tested; Female 1 = 2.92×10^8 copies/mg; Female 2 = not tested). Only the 4.6 kbp fragment was obtained with the PCR directed [18] to the 2.8 kbp insertion, indicating the presence of recombinant MYXV in the tissues of the rabbits and the absence of classical MYXV.

Serology using a commercial kit (Civtest® Cuni Mixomatosis-Hipra) according to the manufacturer's instructions, showed a high antibody titer in the Male rabbit (RI10 of 19.6) and in the Female 2 (RI10 of 9.2), similar to the RI values of hare positive control serum. Despite that the RI value (< 1.0) obtained for the Female 1 suggests no seroconversion, considering that blood serum was not available, no robust conclusions can be taken.

Bordetella bronchiseptica and *Escherichia coli* were isolated from the lungs of the Male and Female 1, respectively. Faeces from rabbits showed small infestations of *Eimeria spp.* oocysts.

3.3. Molecular Characterisation

Around position ≈61,000 nt of the complete MYXV genome (Lausanne strain), ORFs M060R, M061R, M062R, M063R M064R, M065R and M066R are sequentially located and close together, in different frames, with ORFs M065R and M066R overlapping by 100 nt (Figure 5A).

Sequencing analysis of the 4.6 kbp amplicon confirmed the presence of an additional 2.8kbp region within the M009L gene around nucleotide position 12,336 in the reference MYXV strain AF170726. M009L split into ORF M009L-a containing the original 5' end, and ORF M009L-b corresponding to the original 3' end.

In silico analysis of the 4600/2800 bp sequence showed the presence of five ORFs with some degree of similarity with genes M060R, M061R, M064R, M065R and M066R of MYXV strains, but with inverted orientation (Figure 5B).

ORF M066R encodes a 185 aa long protein and is found in MYXV (e.g. AAF14954.1). ORF M066L (the remaining of the complete gene M066R) encodes a putative partial protein of 70 amino acids in the recombinant MYXV from Portugal. Despite being present in MYXV-Tol and ha-MYXV strains from Spain [13,15], this ORF was not annotated previously. M066L is recognised between ORF M065L and ORF M009L-b, sharing 80% similarity with the homologous sequence of ORF M066R in the ha-MYXV. This small ORF overlaps the M009L-b ORF by 21 nucleotides and M065 by 101 nucleotides (Figure 5C).
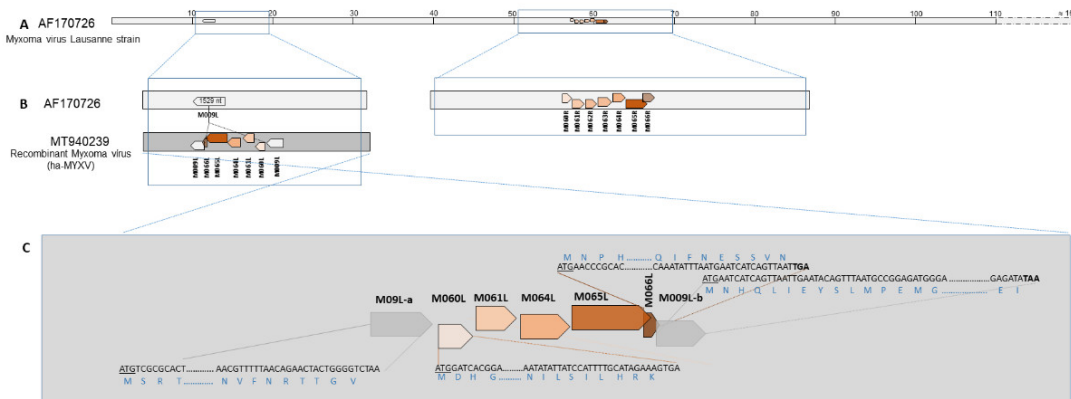
Figure 5. (**A**) Linear genomic organisation of the reference Lausanne strain with the location of ORFs M009L and ORFs M061R and M66R. (**B**) Schematic representation comparing the uninterrupted receptor ORF M009L in the Lausanne strain with the insert in ha-MYXV. (**C**) Detail on the flaking regions of the insert and relative position of the ORFs.

The two nucleotide sequences obtained from the Male and Female 1 wild rabbits were identical to each other and to the homologous sequences from other MYXV-Tol/ha-MYXV (MK836424 and MK340973). The differences between the truncated ORF M066L and the homologous M066R ORFs from MYXV-Tol and ha-MYXV obtained from hares (MK836424 and MK340973), and classical MYXV obtained from rabbits (MK388144, MK388143, MK388142 and MK388141 (MYXV) are shown in Figure S1. In particular, the percentage of similarity between ORF M066L and ORFs M066R despite its species of origin is around 79%.

The M066L sequences obtained are 100% similar to correspondent sequences of recombinant MYXV strains described before (MK836424 and MK340973) in Spain. About 79% of similarity was observed between the M066L and the M066R sequences from other ha-MYXV and MYXV strains.

The putative M066L protein obtained presented 80% identity with M066R protein of MYXV-Tol and ha-MYXV strains described before (MK340973 and MK836424). The same similarity was also observed between the M066L and the M066R sequences from classic rabbit MYXV strains (Figure S2).

3.4. Isolation of the Virus in Cell Culture

The successful isolation of the recombinant MYXV in RK13 cells from a separate eyelid, genitalia and lung samples from the Male rabbit and from the eyelid of the Females rabbits, confirmed its viability and infectiousness, proving that the virus was multiplying in the rabbits' tissues. Viral isolation was confirmed by cytopathic effect (CPE) at day 5 in RK13 subconfluent infected cells, by indirect immunofluorescence of the cells (*protocol available on request*) and by conventional PCR of the cell supernatant.

The characteristic CPE at late stage of infection was observed from day three after inoculation (Figure 6A, C). By qPCR we demonstrated the progressive decrease of the Cq value in DNA samples extracted from cell culture supernatant aliquots, collected at day 1, 5 and 10 *(results not shown)*. To

demonstrate the presence of the viral protein in the RK13 cells, an indirect immunofluorescence test was performed at day 3 (Figure 6B) and day 6 post inoculation, allowing to confirm Myxoma virus foci (Figure 6D).
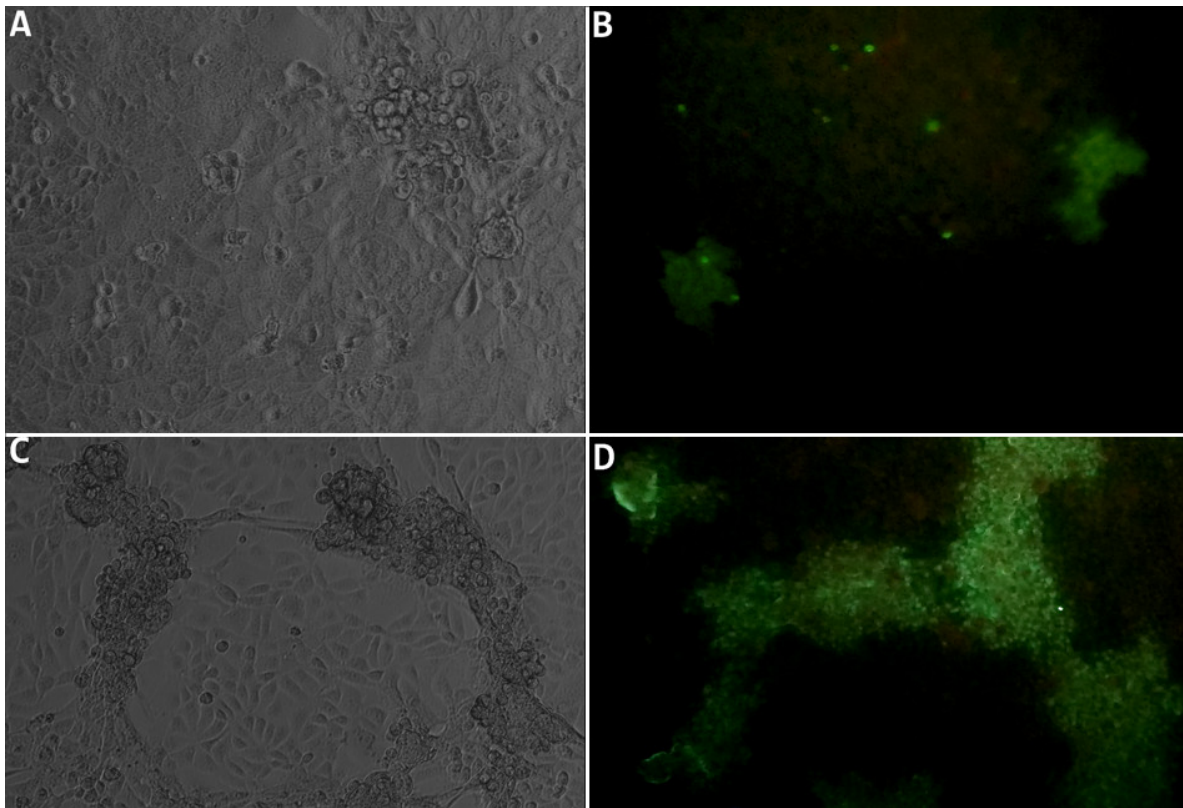
Figure 6. (**A**) Cytopathic effect in RK13 cells infected with an eyelid homogenate of the rabbit Male, three days after the infection, consisting of smaller aggregates of round and refringent cells, surrounded by normal cells (100×). (**B**) Positive indirect immunofluorescence staining (IFI) of recombinant MYXV infected RK13 cells three days after the inoculation (100×) (**C**) Cytopathic effect in RK13 cells infected with an eyelid homogenate of the rabbit male, six days after the infection, consisting of large aggregates of round and refringent cells forming cords over the normal cell layer (100×). (**D**) Positive immunofluorescence staining of the recombinant MYXV infected RK13 cells, six days after the inoculation (100×). Images in B and D (IFI staining) do not correspond to the same zone of the non-stained cells (A and C).

3.5. Transmission Electron Microscopy

Analysis by electron microscopy allowed observing poxvirus-compatible particles in the lung and eyelid. In the eyelid, a higher number of viral particles was observed, especially in epithelial cells (Figure 7). The degree of advanced autolysis of the tissues did not allow a more detailed evaluation of the type of cells mostly infected.
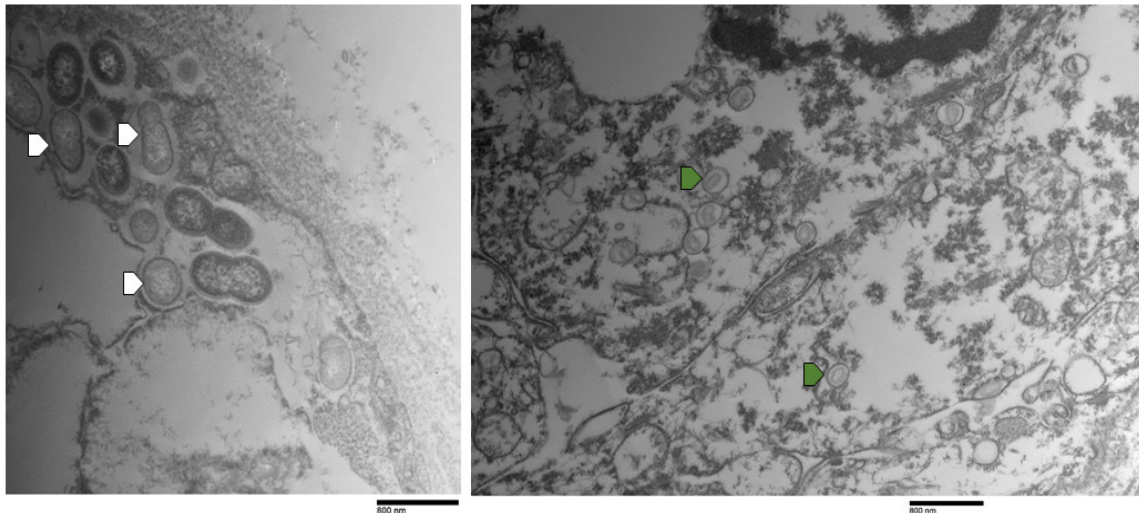
Figure 7. Transmission electron microscopy (TEM) micrographs. On the left, a lung cut from Female 1. At right, an eyelid epithelium cut from Female 1. White arrow heads indicate immature viral particles and green arrow heads indicate apparently mature viral particles. The black bar indicates the scale (800 nm).

4. Discussion

The external signs of myxomatosis in three adult rabbits found dead between June and August 2020 in Alentejo that arrived to the National Reference Laboratory for Animal Diseases (INIAV, I.P.) for investigation corresponded to mild to moderate swelling of the eyelids and genitalia.

Both male and females tested positive to MYXV-DNA by the M000.5 L/R gene qPCR, and by the 2.8 kbp insert PCR, showing infection by the natural recombinant MYXV. None of the rabbits were co-infected with classical MYXV strains, RHDV2, RHDV or LeHV-5.

Most of the wild rabbits that died of myxomatosis, generally arrived to the laboratory with severe swelling of the eyelids and genitals, often accompanied by ocular purulent discharge and very frequently in a state of thinness or cachexia. The high viral loads found in several tissues, and the good body condition of these three wild rabbits, suggest that a shorter course of disease may have taken place. Although further testing is necessary to support this relation, this may imply a possible higher virulence of the natural recombinant MYXV strain towards wild rabbits. With regards to the MYXV strains, the national surveillance plan of wild leporids in action in Portugal since 2017 allowed for the testing of more than 57 infected wild rabbits [22]. A total of 73% of the rabbits found dead with myxomatosis (infected with classic MYXV strains) presented median/poor corporal condition or even cachexia, reflecting the ability of the animals to survive infected for longer periods [22]. A lower adaptation of the recombinant MYXV strains to rabbits, comparing the MYXV classic strains with which rabbits have evolved for more than 50 years [23], may eventually account for these apparent differences.

The detection of a recombinant MYXV circulating in hares, and its apparent segregation from MYXV circulating in rabbits, initially suggested the adaptation of MYXV-Tol and ha-MYXV to hares in order to efficiently multiply in this species.

Sequencing of the 2.8 kbp insert from the two rabbits showed that both recombinant MYXV strains have the same poxvirus gene "cassette" previously described in Iberian hares [3,13,15].

However, we described a putative truncated gene similar to the M066R gene of the myxoma virus that is also present, though not annotated, in the Myxoma virus sequences obtained previously from Iberian hares. As in the MYXV-Tol (MK836424) and ha-MYXV genomes (MK340973), M062R and the M063R are not found in the insert.

The origin of this insert was discussed previously by other authors, and is not a goal of this work. However, the putative protein encoded by ORF M066L is 65.22% to 76.81% similar to homologous ORFs in capripoxviruses, cervidpoxviruses, suipoxviruses, yatapoxviruses but not with the BeAn

58058 virus, appointed previously [13] as a potential donor, or sharing an ancestral donor, of the genetic material found in the insert. On the other hand, the higher similarity of putative protein encoded by M066L with rabbit fibroma virus and with classical Myxoma virus strains, suggests that the insert may have originated from one of these viruses, or a similar virus, not yet described.

During the three months in which the three rabbits were collected, only a small number of found dead rabbits with myxomatosis arrived at the laboratory from mainland Portugal, limiting any inference about the prevalence, frequency and distribution of the recombinant MYXV in the wild. However, since the natural recombinant MYXV emergence in 2018, and according to data collected under the +Coelho project, Beja was the district from which more hares were sampled (52 out of 170) and tested, and was also one of the districts most affected by myxomatosis (34.6% of positivity in the sample).

The detection of the recombinant MYXV in wild rabbits raises serious concerns at different levels, constituting an additional treat to the already fragile wild rabbit, which entered to the IUCN's endangered conservation status last year [20]. If the recombinant MYXV and classical MYXV strains behave as different viruses in rabbit, with no full cross protection between the two, the jump of a recombinant MYXV into the rabbit populations will eventually accelerate the decline of these already diminished wild populations. On the other hand, the fact that the recombinant MYXV affects both the Iberian hare and the wild rabbit, may favour the maintenance of the virus as more hosts are available for virus replication and circulation. The recombinant MYXV may therefore become endemic in the same way that classic strains did, allowing the co-evolution in both species. However, the ability to infect the wild rabbit, may lead the recombinant MYXV to prefer the rabbit host, taking into account the greater dispersion and higher density compared to the Iberian hare, which would facilitate their environment maintenance. Further concerns include the rabbit industry, and the need to evaluate if MYXV or Shope Fibroma virus attenuated vaccines are protective against the recombinant MYXV.

Although vaccination is highly effective in the industry, inducing generally the seroconversion of almost 100% of the animals [24], parenteral vaccination of wild populations is almost impossible. Another major concern arises from the emergence and circulation of this new strain in wild rabbit populations, in which virus-host co-evolution regarding classical MYXV strains occurred over the years [25]. The emergence of new MYXV strains theoretically poses a great risk to the rabbit threatened of extinction.

Since the complete genome sequences were not obtained in this study, there are no certainties that the recombinant MYXV strains found in the three rabbits are identical to MYXV-Tol or ha-MYXV. Therefore, we cannot exclude the existence of other mutations that may have contributed to the ability of the recombinant MYXV to cause disease in rabbits.

5. Conclusions

Almost two years after the emergence of a recombinant MYXV in Iberian hares, our findings bring one new piece into the model of host-myxoma virus co-evolution by demonstrating the pathogenicity of this recombinant virus towards rabbits. It is important to continue monitoring the disease in wild rabbits and hares in order to ascertain the geographic dimension of the spillover phenomena or the spread of this jump of recombinant hare MYXV back to the European rabbit.

Declaration: This study did not use live animals and was carried out within the scope of a National Plan for the Control of Rabbit Haemorrhagic Disease Virus 2 in rabbits (Dispatch no. 4757/2017 of 31 May), with the legal authorisations from the National Authority-the Institute for Nature Conservation and Forests (Instituto da Conservação da Natureza e das Florestas, I.P., ICNF).

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Delibes-Mateos, M.; Delibes, M.; Ferreras, P.; Villafuerte, R. Key role of European rabbits in the conservation of the western Mediterranean Basin hotspot. *Conserv. Biol.* **2008**, *22*, 1106–1117, doi:10.1111/j.1523-1739.2008.00993.x.

2. Staniforth, R.J.; Cavers, P.B. The importance of cottontail rabbits in the dispersal of *Polygonum* Spp. *J. Appl. Ecol.* **1977**, *14*, 261–268.

3. Carvalho, C.L.; Abade dos Santos, F.A.; Fagulha, T.; Carvalho, P.; Mendonça, P.; Monteiro, M.; Duarte, M.D. Myxoma virus and rabbit haemorrhagic disease virus 2 coinfection in a European wild rabbit (*Oryctolagus cuniculus algirus*), Portugal. *Vet. Rec. Case Rep.* **2020**, 18–21, doi:10.1136/vetreccr-2019-001002.

4. Murphy, F.A.; Fauquet, C.M.; Bishop, D.H.L.; Ghabrial, S.A.; Jarvis, A.W.; Martelli, G.P.; Mayo, M.A.; Summers, M. *Virus Taxonomy: Classification and Nomenclature of Viruses*; Springer: Berlin/Heidelberg, Germany, 1995.

5. Sanarelli, G. Das myxomatogene Virus. Beitrag zum Studium der Krankheitserreger ausserhalb der Sichtbaren. (Vorliiufige Mitteilung.). *Zentralbl. Bakteriol. Parasitenkd. Infektionskrankh. Abt. I.* **1898**, *23, 865–873*.

6. Rivers, T.M. Infectious myxomatosis of rabbits. *J. Exp. Med.* **1930**, *51, 965–976*.

7. Marlier, D.; Cassart, D.; Coignoul, F.; Vindevogel, H. Experimental Infection of Specific Pathogen-free New Zealand White Rabbits with Five Strains of Amyxomatous Myxoma Virus. *J. Comp. Pathol.* **1999**, *121*, 369–384.

8. Moss, B. Fields virology. In *Field's Virology*; Knipe, D.M., Howley, P.M., Eds.; Springer: Berlin/Heidelberg, Germany, 2001; pp. 2849–2883.

9. Bertagnoli, S.; Marchandeau, S. Myxomatosis. *Rev. Sci. Tech. Off. Int. Epiz.* **2015**, *34*, 549–556.

10. Arthur, C.P.; Louzis, C. A review of myxomatosis among rabbits in France. *Rev. Sci. Tech. Off. Int. Epiz.* **1988**, *7*, 959–976.

11. Barlow, A.; Lawrence, K.; Everest, D.; Dastjerdi, A.; Finnegan, C.; Steinbach, F. Confirmation of myxomatosis in a European brown hare in Great Britain. *Vet. Rec.* **2014**, *175*, 75–76, doi:10.1136/vr.g4621.

12. Collins, J.J. Myxomatosis in the common hare. *Ir. Vet. J.* **1955**, *9*, 268.

13. Pinto, A.; Matos, A.L. De; Abrantes, M.; Kraberger, S.; Risalde, M.A.; Gort, C.; Mcfadden, G.; Varsani, A.; Esteves, P.J. Genetic Characterization of a Recombinant Myxoma Virus in the Iberian Hare (*Lepus granatensis*). *Viruses* **2019**, *11*, 1–16, doi:10.3390/v11060530.

14. Bocanegra, I.G.; Camacho-Sillero, L.; Risalde, M.A.; Dalton, K.; Caballero-Gómez, J.; Aguero, M.; Zorrila, I.; Gómez-Guillamón, F. First outbreak of myxomatosis in Iberian hares (*Lepus granatensis*). *Transbound. Emerg. Dis.* **2019**, 1–5, doi:10.1111/tbed.13289.

15.　Dalton, K.P.; Martín, J.M.; Nicieza, I.; Podadera, A.; Llano, D.; Casais, R.; Gimenez, S.; Badiola, I.; Agüero, M.; Duran, M.; et al. Myxoma virus jumps species to the Iberian hare. *Transbound. Emerg. Dis.* **2019**, doi:10.1111/tbed.13296.

16.　Carvalho, C.; Abade dos Santos, F.A.; Monteiro, M.; Carvalho, P.; Mendonça, P.; Duarte, M.D. First cases of myxomatosis in Iberian hares (*Lepus granatensis*) in Portugal. *Vet. Rec. Case Rep.* **2020**, *8*, doi:10.1136/vetreccr-2019-001044.

17.　Duarte, M.D.; Barros, S.C.; Henriques, A.M.; Fagulha, M.T.; Ramos, F.; Luís, T.; Fevereiro, M. Development and validation of a real time PCR for the detection of myxoma virus based on the diploid gene M000.5L/R. *J. Virol. Methods* **2013**, *196*, 219–224, doi:10.1016/j.jviromet.2013.11.014.

18.　Duarte, M.D.; Carvalho, C.L.; Barros, S.C.; Henriques, A.M.; Ramos, F.; Fagulha, T.; Luís, T.; Duarte, E.L.; Fevereiro, M. A real time Taqman RT-PCR for the detection of rabbit hemorrhagic disease virus 2 (RHDV2). *J. Virol. Methods* **2015**, *219*, 90–95, doi:10.1016/j.jviromet.2015.03.017.

19.　Abade dos Santos, F.A.; Monteiro, M.; Pinto, A.; Carvalho, C.L.; Peleteiro, M.C.; Carvalho, P.; Mendonça, P.; Carvalho, T.; Duarte, M.D. First description of a herpesvirus infection in genus Lepus. *BioRxiv Prepr.* **2020**, doi: 10.1101/2020.01.21.913723.

20.　Villafuerte, R.; Delibes-Mateos, M. *Oryctolagus cuniculus* (errata version published in 2020). In *IUCN Red List Threatened Species 2019: e.T41291A170619657*; International Union for Conservation of Nature and Natural Resources: Cmbridge, UK, 2019, doi:10.2305/IUCN.UK.2019-3.RLTS.T41291A170619657.en.

21.　Abade dos Santos, F.A.; Santos, N.; Carvalho, C.; Martinez, M.; Gortazar, C.; Garc, I.; Duarte, M.; Alves, P. Serology unveils decades-long contact of the Iberian hare, *Lepus granatensis*, with myxoma or antigenically-related virus. *Authorea* **2020**, doi:10.22541/au.159818365.55937034.

22.　Duarte, M.D.; Carvalho, C.L.; Abade dos Santos, F.A.; Gomes, J.; Alves, P.C.; Esteves, P.J.; Abrantes, J.; Lopes, A.M.; P., M.; Serronha, A.; et al. +Coelho: Avaliação Ecossanitária das Populações Naturais de Coelho Bravo Visando o Controlo da Doença Hemorrágica Viral. *Relatório de Atividades na Época Venatória* **2018,** *1*, 1–156.

23.　Kerr, P.J.; Liu, J.; Cattadori, I.; Ghedin, E.; Read, A.F.; Holmes, E.C. Myxoma virus and the leporipoxviruses: An evolutionary paradigm. *Viruses* **2015**, *7*, 1020–1061, doi:10.3390/v7031020.

24.　Hipra, L. Serological response to myxomatosis vaccination by different inoculation systems on farm rabbits. *World Rabbit Sci.* **2003**, *11*, 145–156.

25.　Best, S.M.; Kerr, P.J. Coevolution of host and virus: The pathogenesis of virulent and attenuated strains of myxoma virus in resistant and susceptible European rabbits. *Virology* **2000**, *267*, 36–48, doi:10.1006/viro.1999.0104.

SHORT COMMUNICATION

Recombinant myxoma virus infection associated with high mortality in rabbit farming (*Oryctolagus cuniculus*)

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Abstract

Myxomatosis is an emergent disease in the Iberian hare, having been considered a rabbit disease for decades. Genome sequencing of the strains obtained from Iberian hares with myxomatosis showed these to be distinct from the classical ones that circulated in rabbits since the virus introduction in Europe, in 1952. The main genomic difference in this natural recombinant hare myxoma virus (ha-MYXV) is the presence of an additional 2.8 kb region disrupting the M009L gene and adding a set of genes homologous to the myxoma virus (MYXV) genes M060R, M061R, M064R, M065R and M066R originated in Poxviruses. After the emergence of this recombinant virus (ha-MYXV) in hares, in the summer of 2019, the ha-MYXV was not detected in rabbit surveys, suggesting an apparent species segregation with the MYXV classic strains persistently circulating in rabbits. Recently, a group of six unvaccinated European rabbits (*Oryctolagus cuniculus cuniculus*) from a backyard rabbitry in South Portugal developed signs of myxomatosis (anorexia, dyspnoea, oedema of eyelids, head, ears, external genitals and anus, and skin myxomas in the base of the ears). Five of them died within 24–48 hr of symptom onset. Molecular analysis revealed that only the recombinant MYXV was present. This is the first documented report of a recombinant hare myxoma virus in farm rabbits associated with high mortality, which increases the concern for the future of both the Iberian hare and wild rabbits and questions the safety of the rabbit industry. This highlights the urgent need to evaluate the efficacy of available vaccines against this new MYXV.

KEYWORDS
European rabbit, ha-MYXV, myxoma virus, myxomatosis, *Oryctolagus cuniculus cuniculus*, rabbit farming, recombinant myxoma virus

1 | INTRODUCTION

The myxoma virus (MYXV) is a *Leporipoxvirus* from the Poxviridae family that causes an infectious and systemic disease, often fatal in the European rabbit, called myxomatosis. This enveloped virus possesses a brick-shaped morphology and a large double-stranded DNA genome, replicating in the cytoplasm of infected cells (Murphy et al., 1995). After the introduction of the virus in France, in 1952, MYXV spread throughout all of Europe, reaching mortalities rates as high as 90% (Fenner & Ratcliffe, 1965). Over the years, the virulence of the strains decreased significantly due to the increase of genetic resistance by the host, development of immunity and

natural selection of less virulent strains (Fenner & Fantini, 1999; Kerr, 2012). Nowadays, the disease persists (Carvalho et al., 2020b, 2020; Duarte et al., 2018) and is considered one of the main reasons behind the fragile conservation status (endangered) of the European rabbit (Villafuerte & Delibes-Mateos, 2019).

The development and production of heterologous and homologous vaccines against myxomatosis in rabbits proved to confer seroconversion in almost 100% of rabbits (Hipra, 2003), and, along with the application of biosafety measures, allowed for the control of the disease in industrial rabbit farming and small rabbitries, contrarily to wildlife.

For almost seven decades, myxomatosis, considered to be a rabbit disease, has only rarely been reported in European brown hares (Collins, 1955; Wibbelt & Frolich, 2005) and, to the best of our knowledge, has never bee reported in the Iberian hare. However, in late 2018, a natural recombinant MYXV (ha-MYXV) emerged in the Iberian hare, leading to the first epidemic outbreaks in this species throughout Spain and Portugal (Bocanegra et al., 2019; Carvalho et al., 2020). The change in host tropism (rabbit to hare) was attributed to a genomic modification comprising a 2.8 kb insertion (Dalton et al., 2019; Pinto et al., 2019) containing genes with homology to the MYXV M060R, M061R, M064R, and M065R and M066R genes (Abade dos Santos et al., 2020), which are located around position 60 kb in the genome. The 2.8 kb insert disrupted the M009L gene located around position 12 kb in the MYXV genome, which became divided in two smaller ORFs, flanking the insert.

Recently, this natural recombinant virus (ha-MYXV) was reported in wild rabbits with myxomatosis (Abade dos Santos et al., 2020). To date, no cases of mortality due to this recombinant MYXV in domestic European rabbits have been documented. The cases of myxomatosis in wild and domestic rabbits continue to be monitored and genotyped in Portugal, under the scope of a national surveillance programme (+Coelho Project, dispatch 4575, 31 May 2017, MAFDR).

Here, we report an outbreak of high mortality (≈83%) in a small backyard rabbitry. Genotyping confirmed the presence of a recombinant virus, similar to the ha-MYXV previously identified in Iberian hares and the absence of classical MYXV strains. None of the rabbits were vaccinated, impeding any inference to the degree of protection against the recombinant MYXV conferred by commercial vaccines. However, the confirmation that ha-MYXV induces severe disease in rabbits, raises new concerns for the wild and domestic leporid species, emphasizing the need for continuous monitoring and genomic characterization of viruses circulating in these species in the Iberia.

Given that hare MYXV is being detected simultaneously in hares and rabbits, a more generalist designation, non-specific to geographic area or species, such as rec-MYXV (for recombinant myxoma virus) may be more appropriate in the future.

2 | MATERIAL AND METHODS

2.1 | Case presentation

In July 2020, a small rabbit farm (Aljustrel, South Portugal) containing one adult male (two years old), two adult males (one year

FIGURE 1 One year adult male (22388PT20). Purulent blepharoconjunctivitis and muzzle oedema.

old, 22388PT20 and 22391PT20), one adult female (one year old, 22390PT20) and two juveniles (5 months old, a male and a female) European rabbits reported signs compatible with myxomatosis. The six rabbits presented clinical signs of disease on the same day, although with different degrees of severity. Admittedly, the animals were not under daily vigilance by the producer.

The two-year-old adult male showed mild signs of illness, with a very slight swelling of the eyelid and slight serous discharge, recovering from disease in around one week. After the clearance of the clinical symptoms, a blood sample was collected from this rabbit by jugular venepuncture (Abade dos Santos et al., 2019) to evaluate the serological response to MYXV infection using the CIVTEST Cuni Myxomatosis Kit (Hipra, Girona, Spain) according to the manufacturer indications. The other five animals died 24–48 hr after the onset of the symptoms, which included oedema of the eyelids (Figure 1), the base of the ears and genitals, dyspnoea and anorexia.

2.2 | Necropsy and histopathology

After death, three (22388PT20, 22390PT20 and 22391PT20) of the five rabbits were necropsied according to routine procedures. Tissue samples were collected for virology (liver, spleen, lung, eyelid and genitalia).

For histopathology, liver, spleen, kidney, eyelid, ear, muzzle and lung fragments were fixated in 10% (w/v) neutral buffered formalin, routinely paraffin-embedded, sectioned at 4 μm, and stained with Haematoxylin and Eosin (H&E).

The other two rabbits were autolysed and therefore only submitted to virological examination using a pool of organs.

2.3 | Molecular analysis

For nucleic acid extraction, fresh samples of liver and spleen, lung, eyelid and genitalia were homogenized at 20% (w/v) with

phosphate-buffered saline and clarified at 3,000 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, Hilden, Germany), according to the manufacturer's protocol.

In the Scope of Project + Coelho, all the rabbits were tested for RHDV, RHDV2 and MYXV due to possible co-infections (Carvalho et al., 2020b, 2020). The rabbits were tested for rabbit haemorrhagic disease virus 2 (RHDV2) by qPCR (Duarte et al., 2015), and for RHDV by conventional PCR (Tham et al., 1999), using the AgPath (Thermo Fisher Scientific, Massachusetts, USA) and One-step RT-PCR kit (Qiagen, Hilden, Germany), respectively. MYXV was initial tested by the qPCR method described by (Duarte et al., 2014), which detects both MYXV and ha-MYXV virus, using the FastStart TaqMan Probe Master Kit (Roche, Mannheim, Germany).

For viral quantification, qPCR was performed in duplicate with three negative controls (water instead of DNA) and a positive control corresponding to a standard concentration of recombinant plasmid containing a fragment of the M000.5 L/R gene. The assay was validated if the positive control was amplified with the expected Cq, no signal was obtained in the negative controls, and the duplicate amplification curves of the sample were similar.

The preliminary analysis for ha-MYXV DNA was performed with the conventional PCR system 9E/9F described by Dalton et al. (Dalton et al., 2019), using the High-Fidelity PCR Master Mix (Roche), according to manufacturer protocol. Amplifications were carried out in a Bio-Rad CFX96™ Thermal Cycler (Bio-Rad Laboratories, Redmond, USA).

The PCR products were visualized in 2% horizontal electrophoresis agarose gels, purified with the NZYGelpure kit (Nzytech, Lisbon, Portugal), and directly sequenced using the ABI Prism BigDye Terminator v3.1 Cycle sequencing kit on a 3130 Genetic Analyser (Applied Biosystems, Foster City, CA, USA). Sequencing of an 859 nt long fragment within the 2.8 kb insert, which includes the partial M064L and M061L genes, was performed with primers designed in this work, namely 1467F (5′- TTCATCGTTTATGGGAAAATCTATG-3′), 1819R (5′- GAGGGGACAGTTATGGATGTAC- 3′), 2028F (5′-AAGATGCGTCTGTGTAACAATCC-3′) and 2325R (5′-AACAATGTATACACTCATGACAGTAC-3′). The nucleotide sequences obtained were assembled using the SeqScape software v2.7 (Applied Biosystems, Foster City, CA, USA) and submitted to GenBank (MT920563 and MT920564).

3 | RESULTS

3.1 | Necropsy and histopathology

The cadavers of all three necropsied rabbits were in good body condition suggesting an acute course of the disease. Necropsy confirmed the presence of 1 cm nodules at the base of the ears in one male rabbit (Figure 1), together with oedema of the nose, lips and

FIGURE 2 One year adult male (22391PT20). Skin nodules on the ear basis (myxomas)
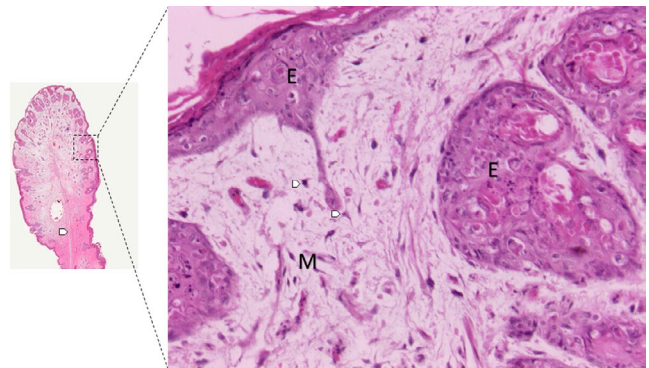
FIGURE 3 Histopathology analysis of rabbit 22391PT20. (Left) nodular thickening corresponding to the accumulation of myxoid tissue in the dermis auricular cartilage (arrowhead, digitalized slide, no magnification, H&E). (Right) Detail of the previous figure in which the myxoid tissue (M) and the star-shaped cells (arrowheads) are visible; epidermis hyperplasia and follicular epithelium (E) (100×, H&E)

external genitalia, purulent blepharitis and lung densification foci. The other adult male showed white ocular discharge (Figure 2) and focal lung congestion. Along with oedema of left eyelid, another rabbit also presented thickening foci of ears.

Histopathology of the skin lesions revealed marked epidermal hyperplasia, frequent ballooning degeneration of keratinocytes and myxoid change of the dermal connective tissue (Figure 3). In the lung, thickening of inter-alveolar septa was observed due to infiltration of heterophils and mononucleated inflammatory cells, confirming interstitial pneumonia (Figure 4).

3.2 | Virological results

All animals tested negative for RHDV and RHDV2. The two-year-old male rabbit was seropositive for MYXV antibodies, showing a high antibody titre of 18.4 (RI10). No serum was available from the five dead rabbits due to blood coagulation.

The Cq values (and correspondent viral load/mg tissue) obtained with the qPCR-MYXV targeting the diploid gene M000.5 L/R in the
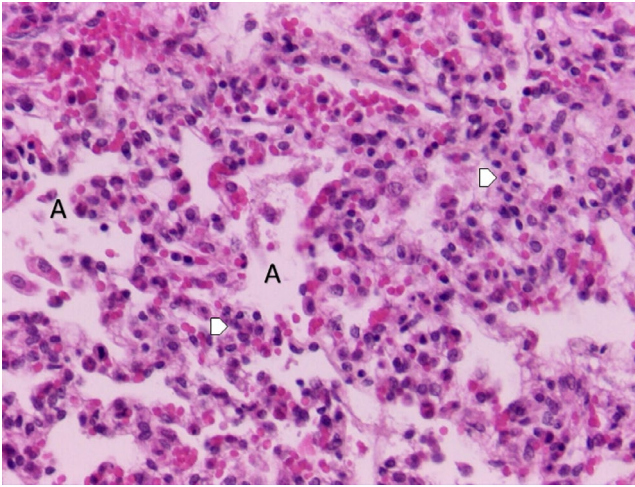
FIGURE 4 Histopathology analysis of rabbit 22388PT20. Thickening of inter-alveolar septa by infiltration of mononucleated cells (arrowheads); pulmonary alveoli (A) (200×, H&E)

22391PT20 (MT920564) revealed 100% of similarity between the two sequences. BLAST analysis showed also 100% of identity with the homologous region encompassing M061L and M064L of sequence MK340973 (ha-MYXV), confirming that rabbits were infected by the recombinant myxoma virus.

4 | DISCUSSION

The presence of external signs of myxomatosis in four adults and two juveniles farm European rabbits during an outbreak registered in Aljustrel (Alentejo, South Portugal) in August 2020; the clinical outcome (83% mortality, 100% morbidity), along with the histopathological and molecular data, confirmed that five out of six infected rabbits died from this disease and that the virus involved was the recombinant myxoma virus (ha-MYXV), thus demonstrating the susceptibility of rabbits to this new virus.

The mortality found in this small backyard rabbitry was apparently higher than what was estimated in previous Iberian hares' outbreaks (55.4%) (García-Bocanegra et al., 2020), although the small number of animals involved may have biased this evaluation.

Lesions described in the three necropsied rabbits, particularly the skin lesions, were fully comparable to the lesions of classical myxomatosis also found in hares (Abade dos Santos et al., 2020; Bocanegra et al., 2019; Carvalho et al., 2020b,2020), such as ballooning degeneration of keratinocytes and myxoid change of the dermal connective tissue.

The viral loads found in these three rabbits are within the ranges seen in other rabbits infected with classic MYXV diagnosed in our laboratory. The values are highest in the eyelids and genitals, as expected, and values about 10–100× higher (comparing with classic

tissues from three rabbits revealed high viral loads in a pool of liver and spleen (1.49E + 07 to 4.48E + 09 copies/mg), lungs (6.44E + 07 to 4.44E + 09 copies/mg), eyelid (2.45E + 10 to 8.45E + 10 copies/mg) and genitalia (1.51E + 09 to 2.48E + 09 copies/mg). The viral load distribution in four different organs is presented in Figure 5. The other two rabbits tested positive for organ pools. The viral loads from five other wild rabbits, diagnosed with classic MYXV strains in our laboratory, are presented in Figure 5 for comparing.

The amplicon size obtained with the 9E/9F system (≈3,100 bp) confirmed that the rabbits were infected with ha-MYXV and not with classic MYXV strains. Further nucleotide sequencing of an 859nt fragment from two rabbits 22390PT20 (MT920563) and

FIGURE 5 Viral loads distribution within different organs. The three rabbits reported in this study (three adults) are represented in orange and compared with five wild rabbits infected with classic MYXV (blue), diagnosed in our laboratory with the same method

MYXV-infected rabbits) in the eyelid of the three rabbits infected with ha-MYXV were found (Figure 5). The absence of published studies describing viral loads in different tissues hampers further comparisons.

Interestingly, the oldest of the rabbits (the 2-year-old male) developed very mild symptoms and survived the infection developing high antibody titre (RI10 = 18.4). The owner reported no previous vaccination but as the oldest animal in the group, we cannot exclude that it may have had previous contact with classic MYXV strains, acquiring some degree of cross-immunity which may have resulted in its survival. This previous exposure may explain the high titre, although it may just be the result of the infection that victimized the co-habitant rabbits. Unfortunately, the commercial kit used to detect antibodies does not distinguish classes of immunoglobulins.

Despite the biosecurity measures adopted in the rabbit industry, introduction of a virus from the outside is always at risk. The rabbit industry is one of the largest expanding meat industries in the world, having had a growth of 85% between 1998 and 2017 (Trocino et al., 2019). In fact, around 1,483,000 tons of rabbit meat was produced in the world in 2017 (Trocino et al., 2019), China, Democratic People's Republic of Korea and Spain being the bigger producers. In China, the research funding for rabbit science reaches values of 2,443 million of Euros, and in France and Germany, the research in this area is funded with around 600–700 millions of Euros a year (Trocino et al., 2019).

From the emergence of myxomatosis in Europe around the 1950s until the development of vaccines, millions of domestic rabbits have died resulting in incalculable economic losses. In Great Britain alone, tens of millions of rabbits have died (Bartrip, 2008). Spillover between the wild and domestic animals has been pointed out as the source of infection for outbreaks in the industry (Carvalho et al., 2017). Given the impact that viral infectious diseases may have in this sector, it is critical to monitor the sanitary status of the industry and the wild populations.

Very recently, cases of disease and mortality from myxomatosis in wild rabbit have spread in southern Portugal (Abade dos Santos et al., 2020), and ha-MYXV DNA (García-Bocanegra et al., 2020) has been found in two rabbits in Spain. All these reports suggest that the virus is infecting the rabbit frequently, which increases the probability of its maintenance, and to become responsible for more outbreaks in European rabbit and Iberian hare.

To the best of our knowledge, this is the first documented description of myxomatosis in domestic rabbits by recombinant MYXV (also referred to as ha-MYXV or MYXV-Tol) infection. It is crucial to verify whether commercial vaccines protect rabbits effectively against this recombinant virus. It is also important to monitor the spread of this recombinant virus to the populations of wild and domestic rabbits to understand whether it has survival advantages over and pathological differences to the classical strains.

5 | DATA AVAIALABILITY STATEMENT

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

CONFLICT OF INTEREST

The authors declare no competing interests.

ETHICAL APPROVAL

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 the purpose of this study.

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REFERENCES

Abade dos Santos, F. A., Carvalho, C. L., Peleteiro, M. C., Gabriel, S., Patrício, R., Abade, F. A., Carvalho, C. L., Peleteiro, M. C., Gabriel, S. I., Patrício, R., Carvalho, J., Cunha, M. V., & Duarte, M. D. (2019). Blood collection from the external jugular vein of Oryctolagus cuniculus algirus sedated with midazolam : Live sampling of a subspecies at risk Blood collection from the external jugular vein of Oryctolagus cuniculus algirus sedated with midazolam : Live. *Wildlife Biology, 1*, 1–10. https://doi.org/10.2981/wlb.00588.

Abade dos Santos, F., Carvalho, C., Pinto, A., Rai, R., Monteiro, M., Carvalho, P., Mendonça, P., Peleteiro, M. C., Parra, F., & Duarte, M. D. (2020). Detection of recombinant hare myxoma virus in wild rabbits (Oryctolagus cuniculus algirus). *Viruses, 12*, 1–12. https://doi.org/10.3390/v12101127

Bartrip, P. W. J. (2008). Myxomatosis in 1950s Britain. *Twentieth Century British History, 19*, 83–105. https://doi.org/10.1093/tcbh/hwm016

Bocanegra, I. G., Camacho-Sillero, L., Risalde, M. A., Dalton, K., Caballero-Gómez, J., Aguero, M., Zorrila, I., & Gómez-Guillamón, F. (2019). First outbreak of myxomatosis in Iberian hares (Lepus granatensis). *Transboundary and Emerging Diseases, 66*(6), 1–5, https://doi.org/10.1111/tbed.13289

Carvalho, C. L., Abade dos Santos, F. A., Fagulha, T., Carvalho, P., Mendonça, P., Monteiro, M., & Duarte, M. D. (2020). *Myxoma virus and rabbit haemorrhagic disease virus 2 coinfection in a European wild rabbit (Oryctolagus cuniculus algirus), Portugal*. 18–21, https://doi.org/10.1136/vetreccr-2019-001002

Carvalho, C., Abade Dos Santos, F. A., Monteiro, M., Carvalho, P., Mendonça, P., & Duarte, M. D. (2020). First cases of myxomatosis in Iberian hares (Lepus granatensis) in Portugal. *Veterinary Record Case Reports, 8*, https://doi.org/10.1136/vetreccr-2019-001044.

Carvalho, C. L., Leclerc Duarte, E., Monteiro, J. M., Afonso, C., Pacheco, J., Carvalho, P., Mendonça, P., Botelho, A., Albuquerque, T., Themudo,

P., Fevereiro, M., Henriques, A. M., Santos Barros, S., & Dias Duarte, M. (2017). Progression of rabbit haemorrhagic disease virus 2 upon vaccination in an industrial rabbitry: A laboratorial approach. *World Rabbit Science*, *25*, 73–85. https://doi.org/10.4995/wrs.2017.5708

Collins, J. J. (1955). Myxomatosis in the common hare. *Irish Veterinary Journal*, *9*, 268.

Dalton, K. P., Martín, J. M., Nicieza, I., Podadera, A., Llano, D., Casais, R., Gimenez, S., Badiola, I., Agüero, M., Duran, M., Buitrago, D., Romero, L. J., García, E., & Parra, F. (2019). *Myxoma virus* jumps species to the Iberian hare. *Transboundary and Emerging Diseases*, *66*, 2218–2226. https://doi.org/10.1111/tbed.13296.

Duarte, M. D., Barros, S. C., Henriques, A. M., Fagulha, M. T., Ramos, F., Luís, T., & Fevereiro, M. (2014). Development and validation of a real time PCR for the detection of myxoma virus based on the diploid gene M000. 5L / R. *Journal of Virological Methods*, *196*, 219–224. https://doi.org/10.1016/j.jviromet.2013.11.014

Duarte, M. D., Carvalho, C. L., Abade dos Santos, F. A., Gomes, J., Alves, P. C., Esteves, P. J., Abrantes, J., Lopes, A. M., Cunha, M. V. (2018). *+Coelho: Avaliação Ecossanitária das Populações Naturais de Coelho Bravo Visando o Controlo da Doença Hemorrágica Viral.*

Duarte, M. D., Carvalho, C. L., Barros, S. C., Henriques, A. M., Ramos, F., Fagulha, T., Luís, T., Duarte, E. L., & Fevereiro, M. (2015). A real time Taqman RT-PCR for the detection of rabbit hemorrhagic disease virus 2 (RHDV2). *Journal of Virological Methods*, *219*, 90–95. https://doi.org/10.1016/j.jviromet.2015.03.017

Fenner, F., & Fantini, B. (1999). History of Myxomatosis - an Experiment in Evolution. In: *Biological Control of Vertebrate Pests*, (pp. 1–339). New York, NY: CABI.

Fenner, F., & Ratcliffe, F. N. (1965). *Myxomatosis*. Cambridge Univ. Press.

García-Bocanegra, I., Camacho-Sillero, L., Caballero-Gómez, J., Aguero, M., Gómez-Guillamón, F., Ruiz-Casas, J., Díaz-Cao, J., García, E., Ruano, M., & Haza, R. (2020). Monitoring of emerging myxoma virus epidemics in Iberian hares. *Transboundary and Emerging Diseases*. *00*, 1–8. https://doi.org/10.1111/tbed.13781.

Hipra, L. (2003) *Serological response to myxomatosis vaccination by different inoculation systems on farm rabbits.* 145–156.

Kerr, P. J. (2012). Myxomatosis in Australia and Europe: A model for emerging infectious diseases. *Antiviral Research*, *93*, 387–415. https://doi.org/10.1016/j.antiviral.2012.01.009

Murphy, F. A., Fauquet, C. M., Bishop, D. H. L., Ghabrial, S. A., Jarvis, A. W., Martelli, G. P., Mayo, M. A., & Summers, M. (1995). *Virus taxonomy: Classification and nomenclature of Viruses.* Springer Verlag, Vienna, Austria: Sixth report of the International Committee for the Taxonomy of Viruses. Archives of Virology, Suppl. 10.

Pinto, A., De Matos, A. L., Abrantes, M., Kraberger, S., Risalde, M. A., Gort, C., Mcfadden, G., Varsani, A., & Esteves, P. J. (2019). Genetic characterization of a recombinant myxoma virus in the Iberian Hare (Lepus granatensis). *Viruses*, *11*, 1–16. https://doi.org/10.3390/v11060530

Tham, K. M., Barnes, S. M., & Hunter, S. N. (1999). Polymerase chain reaction amplification and gene sequence analysis of a calicivirus from a feral rabbit. *Virus Genes*, *18*, 235–242.

Trocino, A., Cotozzolo, E., Zomeño, C., Petracci, M., Xiccato, G., Castellini, C., Trocino, A., Cotozzolo, E., Zomeño, C., & Petracci, M. (2019). Rabbit production and science : The world and Italian scenarios from 1998 to 2018. *Italian Journal of Animal Science*, *18*, 1361–1371. https://doi.org/10.1080/1828051X.2019.1662739

Villafuerte, R., & Delibes-Mateos, M. (2019). *Oryctolagus cuniculus (errata version published in 2020).* IUCN Red List Threat. Species 2019 e.T41291A170619657. https://doi.org/10.2305/IUCN.UK.2019-3.RLTS.T41291A170619657.en

Wibbelt, G., & Frolich, K. (2005). Infectious diseases in European brown hare (lepus europaeus). *Wildlife Biology in Practice*, *1*, 86–93. https://doi.org/10.2461/wbp.2005.1.11.

Can a leporid be infected by more than one strain of MYXV?

Scientific publication 12

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

■■

Both Iberian hare and wild rabbit may be coinfected by MYXV and ha-MYXV. The virulence of these strains towards rabbits and hares appears to be different.

Transboundary and Emerging Diseases

WILEY

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 'CGGTACATCG-TACGCACAC'.

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 fol-lowed 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
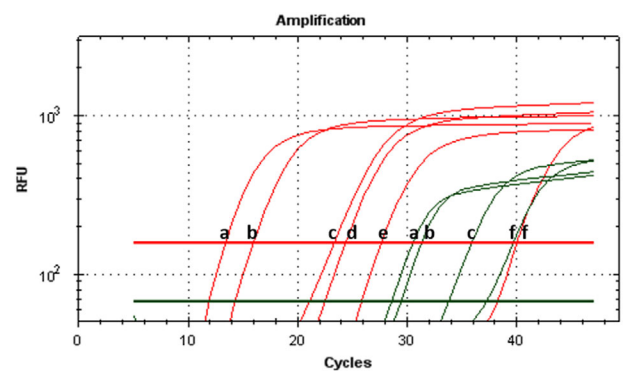
FIGURE 1 Multiplex real-time PCR amplification curves of rabbit-1 and hare-1. Red curves (TexasRed channel) correspond to amplification with the M060L system, and green curves (HEX channel) correspond to amplification with the M009L system. Rabbit-1 samples: eyelid (a); external genitalia (b); spleen (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.
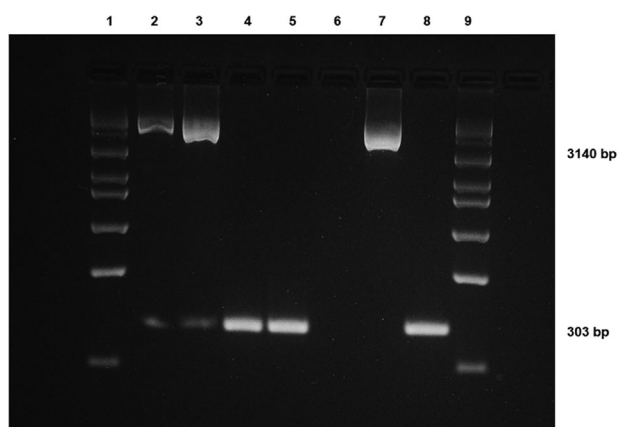
FIGURE 2 Agarose gel electrophoresis showing the amplification products obtained with the seq9E/seq9F PCR system. Lane 1–DNA markers. Lane 2–rabbit-1 (eyelid), extension of 3 min; Lane 3–hare-1 (eyelid), extension of 3 min; Lane 4–rabbit-1 (eyelid), extension of 15 s; Lane 5–hare-1 (eyelid), extension 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
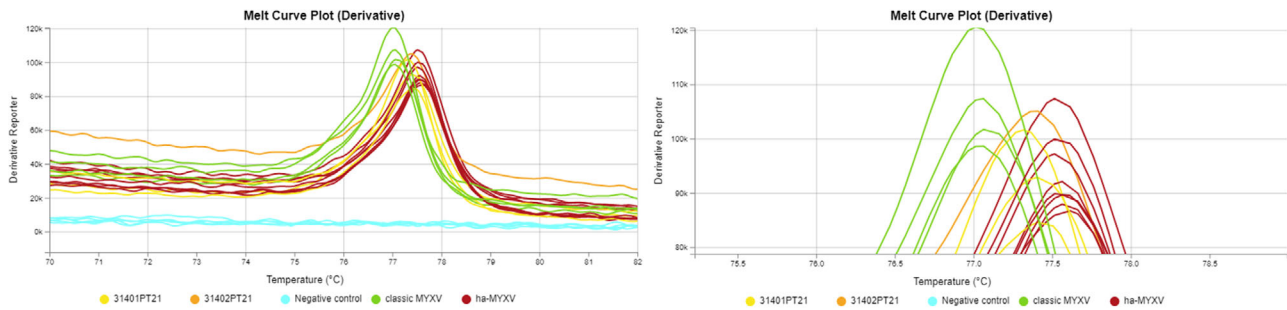
FIGURE 3 (Left) Melt curve plot (derivative) of negative controls (blue curves) classic MYXV strains (green curves), ha-MYXV strains (red curves) and the two co-infected animals 31401PT21 (yellow curves) and 31402PT21 (orange curves). (Right) Higher magnification of the peaks of the three types of samples

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).

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.

REFERENCES

Abade dos Santos, F., Carvalho, C., Monteiro, M., Carvalho, P., Mendonça, P., Peleteiro, M. C., & Duarte, M. D. (2020). Recombinant myxoma virus infection associated with high mortality in rabbit farming (*Oryctolagus cuniculus*). *Transboundary and Emerging Diseases*, 68(4), 2616–2621. https://doi.org/10.1111/tbed.13899

Abade dos Santos, F., Carvalho, C., Pinto, A., Rai, R., Monteiro, M., Carvalho, P., Mendonça, P., Peleteiro, M. C., Parra, F., & Duarte, M. D. (2020).

Detection of recombinant hare myxoma virus in wild rabbits (*Oryctolagus cuniculus algirus*). *Viruses*, *12*, 1–12, https://doi.org/10.3390/v12101127

Abade dos Santos, F. A., Carvalho, C. L., Parra, F., Dalton, K. P., &Peleteiro, M. C. (2021). A quadruplex qPCR for detection and differentiation of classic and natural recombinant myxoma virus strains of leporids. *International Journal of Molecular Sciences*, *22*(1), 1–19.

Abade Dos Santos, F. A., Carvalho, C. L., Peleteiro, M. C., Parra, F., & Duarte, M. D. (2021). A versatile qPCR for diagnosis of leporid gammaherpesvirus 5 using Evagreen® or Taqman® Technologies. *Viruses*, *13*(4), 715. https://doi.org/10.3390/v13040715

Abade dos Santos, F. A., Monteiro, M., Pinto, A., Carvalho, C. L., Peleteiro, M. C., Carvalho, P., Mendonça, P., Carvalho, T., & Duarte, M. D., (2020). First description of a herpesvirus infection in the genus *Lepus*. *Plos One*, *15*, e0231795. https://doi.org/10.1371/journal.pone.0231795

Abade dos Santos, F. A., Carvalho, C. L., Valente, P. C. L. G., Armés, H., Reemers, S. S., Peleteiro, M. C., Calonge Sanz, I., Dalton, K. P., Parra, F., & Duarte, M. D. (2022). Evaluation of commercial myxomatosis vaccines against recombinant myxoma virus (ha-MYXV) in Iberian hare and wild rabbit. *Vaccines*, *10*(3), 356. https://doi.org/10.3390/vaccines10030356

Boni, M. F., Lemey, P., Jiang, X., Lam, T. T. -Y., Perry, B. W., Castoe, T. A., Rambaut, A., & Robertson, D. L. (2020). Evolutionary origins of the SARS-CoV-2 sarbecovirus lineage responsible for the COVID-19 pandemic. *Nature Microbiology*, *5*, 1408–1417. https://doi.org/10.1038/s41564-020-0771-4

Carvalho, C. L., Abade dos Santos, F. A., Monteiro, M., Carvalho, P., Mendonça, P., & Duarte, M. D. (2020). First cases of myxomatosis in Iberian hares (*Lepus granatensis*) in Portugal. *Veterinary Record Case Reports*, *8*, 1–5, https://doi.org/10.1136/vetreccr-2019-001044

Carvalho, C. L., Abade dos Santos, F. A., Fagulha, T., Carvalho, P., Mendonça, P., Monteiro, M., & Duarte, M. D. (2020). Myxoma virus and rabbit haemorrhagic disease virus 2 coinfection in a European wild rabbit (*Oryctolagus cuniculus algirus*), Portugal. *Veterinary Record Case Reports*, *8*(1):e001002. https://doi.org/10.1136/vetreccr-2019-001002

Collins, J. J. (1955). Myxomatosis in the common hare. *Irish Veterinary Journal*, *9*, 268.

Dalton, K. P., Martín, J. M., Nicieza, I., Podadera, A., Llano, D., Casais, R., Gimenez, S., Badiola, I., Agüero, M., Duran, M., Buitrago, D., Romero, L. J., García, E., & Parra, F. (2019). Myxoma virus jumps species to the Iberian hare. *Transboundary and Emerging Diseases*, *66*(6), 2218–2226. https://doi.org/10.1111/tbed.13296

Duarte, M. D., Carvalho, C. L., Abade, F. A., dos Santos, J., Gomes, P. C., Alves, P. J., Esteves, J., Abrantes, A. M., Lopes, M. P., Serronha, A., Santos, N., Santos, P. T., Vaz, Y., Carvalho, J., Pinto, F. C., & Amaro, J. (2021). +*Coelho 2: Desenvolvimento e implementação de medidas práticas impulsionadoras da recuperação dos leporídeos silvestres em Portugal*. Fundo Florestal Permanente, Projeto nr 2019014300001.

Duarte, M. D., Carvalho, C. L., Abade, F. A., dos Santos, J., Gomes, P. C., Alves, P. J., Esteves, J., Abrantes, A. M., Lopes, M. P., Serronha, A., Santos, N., Santos, P. T., Vaz, Y., Carvalho, J., Pinto, F. C., Amaro, J., & Cunha, M. V. (2018). +*Coelho: Avaliação Ecossanitária das Populações Naturais de Coelho Bravo Visando o Controlo da Doença Hemorrágica Viral* Fundo Florestal Permanente, 2017014300001.

Duarte, M. D., Carvalho, C. L., Barros, S. C., Henriques, A. M., Ramos, F., Fagulha, T., Luís, T., Duarte, E. L., & Fevereiro, M. (2015). A real time Taqman RT-PCR for the detection of rabbit hemorrhagic disease virus 2 (RHDV2). *Journal of Virological Methods*, *219*, 90–95. https://doi.org/10.1016/j.jviromet.2015.03.017

Fenner, F., & Ratcliffe, F. N. (1965). *Myxomatosis*. Cambridge University Press.

García-Bocanegra, I., Camacho-Sillero, L., Caballero-Gómez, J., Aguero, M., Gómez-Guillamón, F., Ruiz-Casas, J., Díaz-Cao, J., García, E., Ruano, M., & Haza, R. (2020). Monitoring of emerging myxoma virus epidemics in Iberian hares. *Transboundary and Emerging Diseases*, *68*(3), 1275–1282, https://doi.org/10.1111/tbed.13781

García-Bocanegra, I., Camacho-Sillero, L., Risalde, M. A., Dalton, K., Caballero-Gómez, J., Aguero, M., Zorrila, I., & Gómez-Guillamón, F. (2019). First outbreak of myxomatosis in Iberian hares (*Lepus granatensis*). *Transboundary and Emerging Diseases*, *66*(6), 2204–2208. https://doi.org/10.1111/tbed.13289

ICTV (2011). *ICTV 9th report (2011)*. https://talk.ictvonline.org/ictv-reports/ictv_9th_report/dsdna-viruses-2011/w/dsdna_viruses/74/poxviridae

Myers, C. A., Kasper, M. R., Yasuda, C. Y., Savuth, C., Spiro, D. J., Halpin, R., Faix, D. J., Coon, R., Putnam, S. D., Wierzba, T. F., & Blair, P. J. (2011). Dual infection of novel influenza viruses A/H1N1 and A/H3N2 in a cluster of Cambodian patients. *American Journal of Tropical Medicine and Hygiene*, *85*, 961–963, https://doi.org/10.4269/ajtmh.2011.11-0098

Pinto, A., De Matos, A. L., Abrantes, M., Kraberger, S., Risalde, M. A., Gort, C., Mcfadden, G., Varsani, A., & Esteves, P. J. (2019). Genetic characterization of a recombinant myxoma virus in the Iberian hare (*Lepus granatensis*). *Viruses*, *11*, 1–16, https://doi.org/10.3390/v11060530

Tham, K. M., Barnes, S. M., & Hunter, S. N. (1999). Polymerase chain reaction amplification and gene sequence analysis of a calicivirus from a feral rabbit. *Virus Genes*, *18*, 235–242.

Villafuerte, R., Delibes-Mateos, M., (2019). *Oryctolagus cuniculus (errata version published in 2020). IUCN Red List Threat. Species 2019 e.T41291A170619657*. https://doi.org/10.2305/IUCN.UK.2019-3.RLTS.T41291A170619657.en

Wibbelt, G., & Frolich, K. (2005). Infectious diseases in European brown hare (*Lepus europaeus*). *Wildlife Biology in Practice*, *1*, 86–93. https://doi.org/10.2461/wbp.2005.1.11

How effective are commercial vaccines against the new naturally recombinant virus?

Scientific publication 13

Evaluation of Commercial Myxomatosis Vaccines against Recombinant Myxoma Virus (ha-MYXV) in Iberian Hare and Wild Rabbit

■■

In the conditions recommended by the manufactures, commercial vaccines for rabbits are not effective in protecting Iberian hares against myxomatosis. However, higher doses are promising as successful prophylactic tools against the disease.

Article

Evaluation of Commercial Myxomatosis Vaccines against Recombinant Myxoma Virus (ha-MYXV) in Iberian Hare and Wild Rabbit

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Abstract: The recent emergence of a new myxoma virus capable of causing disease in the Iberian hare (*Lepus granatensis*) has resulted in numerous outbreaks with high mortality leading to the reduction, or even the disappearance, of many local populations of this wild species in the Iberian Peninsula. Currently, the available vaccines that prevent myxomatosis in domestic rabbits caused by classic strains of myxoma virus have not been assessed for use in Iberian hares. The main objective of this study was to evaluate the efficacy of commercial rabbit vaccines in Iberian hares and wild rabbits against the natural recombinant myxoma virus (ha-MYXV), bearing in mind its application in specific scenarios where capture is possible, such as genetic reserves. The study used a limited number of animals (pilot study), 15 Iberian hares and 10 wild rabbits. Hares were vaccinated with Mixohipra-FSA vaccine (Hipra) and Mixohipra-H vaccine (Hipra) using two different doses, and rabbits were vaccinated with the Mixohipra-H vaccine or the Nobivac Myxo-RHD PLUS (MSD Animal Health) using the recommended doses for domestic rabbits. After the vaccination trials, the animals were challenged with a wild type strain of ha-MYXV. The results showed that no protection to ha-MYXV challenge was afforded when a commercial dose of Mixohipra-FSA or Mixohipra-H vaccine was used in hares. However, the application of a higher dose of Mixohipra-FSA vaccine may induce protection and could possibly be used to counteract the accelerated decrease of wild hare populations due to ha-MYXV emergence. The two commercial vaccines (Mixohipra-H and Nobivac Myxo-RHD PLUS) tested in wild rabbits were fully protective against ha-MYXV infection. This knowledge gives more insights into ha-MYXV management in hares and rabbits and emphasises the importance of developing a vaccine capable of protecting wild populations of Iberian hare and wild rabbit towards MYXV and ha-MYXV strains.

Keywords: myxomatosis; Iberian hare; European wild rabbit; vaccines; ha-MYXV

1. Introduction

In 2018, a natural recombinant myxoma virus (referred to as ha-MYXV or MYXV-Tol08/18) emerged in the Iberian hare (*Lepus granatensis*), affecting many populations in Spain [1,2] and Portugal [3–5].

With an apparent mortality rate of 55.4% [6], the geographic spread of ha-MYXV outbreaks increased concerns about the resilience limit of the Iberian hare wild populations against the many insidious factors that have accelerated their decline over the last decades [5]. Despite the conservation status of the Iberian hare (*Lepus granatensis*) being considered of "Least Concern" by the IUCN in 2019, many local populations are currently threatened, as a result of severe loss of habitat imposed by human activities, and more recently, due to the emergence of infectious diseases such as ha-MYXV and LeHV-5 [2–5] and the cumulative effects with other diseases such as cysticercosis [5].

Although ha-MYXV was initially detected only in Iberian hares, it was later (mid-2020) reported in wild and domestic rabbits [7,8]. The recognition that ha-MYXV affects not only hares, but also the European rabbit (*Oryctolagus cuniculus*), questions the efficacy of cross-protection conferred by classic field strains of MYXV that circulate in wild rabbits. Likewise, the effectiveness of the commercial vaccines developed to protect domestic rabbits against classic myxoma strains is still unknown with regard to infection with ha-MYXV.

The susceptibility of the European rabbit to ha-MYXV also escalated the previous concerns that in 2019 gave the wild European rabbit (*Oryctolagus cuniculus*), for the first time in history, the status of "Endangered of Extinction", by the IUCN [9,10].

Several strategies have been attempted to protect and recover the native wild rabbit populations for conservation and hunting purposes, such as captive breeding, re-introduction, restocking programs and vaccination campaigns, reviewed in [11].

This analysis constitutes a pilot study using a small number of wild animals to assess the commercial vaccines Mixohipra-FSA and Mixohipra-H as prophylactic tools to protect Iberian hare against ha-MYXV, bearing in mind its use in captive populations. The present study also evaluated the protection conferred by commercial vaccines Mixohipra-H and Nobivac Myxo-RHD PLUS against ha-MYXV in wild European rabbit. The possibility of using these vaccines as prophylactic tools in wild leporids whenever possible may be crucial to ensure the preservation of the genetic viability of the species in Portugal and Spain, particularly in case of an aggravation of the actual sanitary situation. This study also intends to test the susceptibility of wild rabbit to ha-MYXV isolated from the Iberian hare.

This study puts into practice the Measure 7.6 of Project + Coelho 2, entitled "Evaluation of the efficacy of commercial vaccines against myxomatosis in Iberian hare", identified within the National Plan for the control of Rabbit Haemorrhagic Disease 2 in rabbits (Dispatch 4757/17, 31 may ordered by the Minister of Agriculture), as it is vital to test all available resources in the fight against this emergent virus. This study, therefore, constitutes a pivotal step by assessing the potential of commercially available vaccines for the protection of wild Iberian hare and European rabbit.

2. Materials and Methods

2.1. Origin of Animals and Pre-Adaptation to Captivity Conditions

The main study (study 1) used nine 9-month-old, MYXV antibody seronegative, male hares (*Lepus granatensis*) randomly divided into 3 groups. These animals were the first generation born in captivity and were maintained in semi-extensive conditions for the sole purpose of this study. Their progenitors were captured in South Portugal between 31 August 2019 and 9 January 2020. Capture and accommodation of the hares were approved by the Institute for the Conservation of Nature and Forests (ICNF, I.P.), the Portuguese National Authority for Nature Conservation. A MYXV-seropositive, two-year old adult male hare, was used as control of the natural humoral response efficacy against the ha-MYXV.

A total of 10 wild rabbits (*Oryctolagus cuniculus algirus*), 6 months of age, seronegative for antibodies against MYXV were used in this study (study 2). The animals were obtained

from a wild rabbit reproduction farm, where they were kept in captivity under extensive farming (approved by the National Authority (ICNF, I.P.).

Separately (study 3), after the conclusion of studies 1 and 2, an additional group of five 5-month-old hares, MYXV-seronegative, born in captivity after the start of the first trial, 3 females (#013, #014 and #020) and 2 males (#021 and #025), were used to further confirm the results obtained in study 1.

To avoid the effects of captivity stress on the immune response triggered by vaccination, the animals were adapted through a 40-day quarantine in specifically designed and constructed cages (structure description available upon request). These species-specific cages had different dimensions and particularities to allow the expression of natural hare behaviour (grooming, station position, etc.) as much as possible, to minimize injuries and to allow safe handling during the introduction and removal of the animal from the cage. The hare cages included an additional closed, opaque, confined space, to provide refuge and allow the animals to remain quiet.

A subjective rating of the animals' behaviour was scaled from 1 to 3. A rating of 1 indicates a reduced reluctance to handling and sample collection, a rating of 2 indicates a reluctance to initial handling but an absence of sudden movements and a rating of 3 indicates reluctance throughout the entire procedure with many sudden movements.

The animals were observed daily for food, water intake and behaviour through uninterrupted monitoring cameras to assess the signs of disease or stress. The rabbit watering and feeding system did not allow the individual quantitative analysis of drinking water and daily food intake. The animals were acquainted with the staff (4 veterinarians) that carried out the trial. All the animals were subjected to hemogram and biochemical tests, faecal and blood parasite analyses before the study. The animals were considered fit for study when no signs of stress such as weight loss or signs of illness were observed and normal behaviour was maintained.

2.2. Vaccines

Three commercial vaccines against myxomatosis were used: (i) Mixohipra-FSA (HIPRA Headquarters, Amer, Girona, Spain; lot 12M9J), a live heterologous attapulgite adjuvanted vaccine containing Shope fibroma virus; (ii) Mixohipra-H (HIPRA Headquarters, Amer, Girona, Spain; lot 05D7G), a live homologous vaccine containing attenuated myxoma virus; (iii) Nobivac Myxo-RHD PLUS (MSD Animal Health, Boxmeer, The Netherlands; lot A003B02), a live homologous vector vaccine containing two attenuated recombinant myxoma virus vectors expressing the VP60 gene of RHDV or RHDV2 [12].

The investigational veterinary products (IVP) were used within the stated shelf-life.

2.3. Experimental Design

The hares of study 1 were randomly divided into four groups and identified with an earring according to the following:

(i)   H-G1: a negative control group of three animals (#231, #232 and #233), MYXV seronegative and non-vaccinated;

(ii) H-G2: a group of three animals (#076, #077 and #078), MYXV seronegative, vaccinated with a 2.90×10^4 ffu (focus-forming units) dose of Mixohipra-FSA vaccine at the start of the study and 21 days later with a 1.95×10^4 ffu dose of Mixohipra-H vaccine;

(iii) H-G3: a group of three animals (#042, #043 and #044), MYXV seronegative, vaccinated with a 2.90×10^5 ffu dose of Mixohipra-FSA vaccine at the start of the study and 21 days later a 1.95×10^5 dose of Mixohipra-H vaccine;

(iv) H-G4: a positive control animal (#10), MYXV seropositive, collected from the field after natural recovery from myxomatosis.

The wild rabbits (study 2) were randomly divided into three groups and identified with an earring according to the following:

(i)   R-G1: a negative control group of four animals (#449, #451, #000 and #001), MYXV seronegative and non-vaccinated;

(ii)   R-G2: a group of three animals (#442, #444 and #445), MYXV seronegative, vaccinated with a 1.95×10^4 ffu dose of Mixohipra-H vaccine at the start of the study;

(iii)   R-G3: a group of three animals (#446, #447 and #448), MYXV seronegative, vaccinated with a single dose of Nobivac Myxo-RHD PLUS vaccine ($10^{3.0}$–$10^{5.8}$ ffu of each two vectors) at the start of the study.

A schematic overview of the trials is depicted in Figure 1. The vaccines were inoculated subcutaneously (s.c.) with the volume of the vaccine distributed in two different places in the dorsal cervical area. At the time of vaccination, the groups of non-vaccinated animals (#H-G1, H-G4 and R-G1) were inoculated s.c. with 1 mL of sterile phosphate-buffered saline (PBS) pH 7.2.

The additional group of five 5-month-old hares (study 3) was vaccinated as group H-G3, with an infectious dose of 2.90×10^5 ffu of Mixohipra-FSA vaccine and 21 days later revaccinated with Mixohipra-FSA vaccine with the same infectious dose (2.90×10^5 ffu).

The hare and rabbit experimental trials were carried out separately. Between experiments, the installations were cleaned and disinfected, submitted to 24 h of UVC irradiation, and subjected to a 60-day sanitary vacuum. During these two trials, blood samples were collected from all animals according to the protocol represented in Figure 1. At the time of sampling, body weight, respiratory rate and rectal temperature were also monitored.
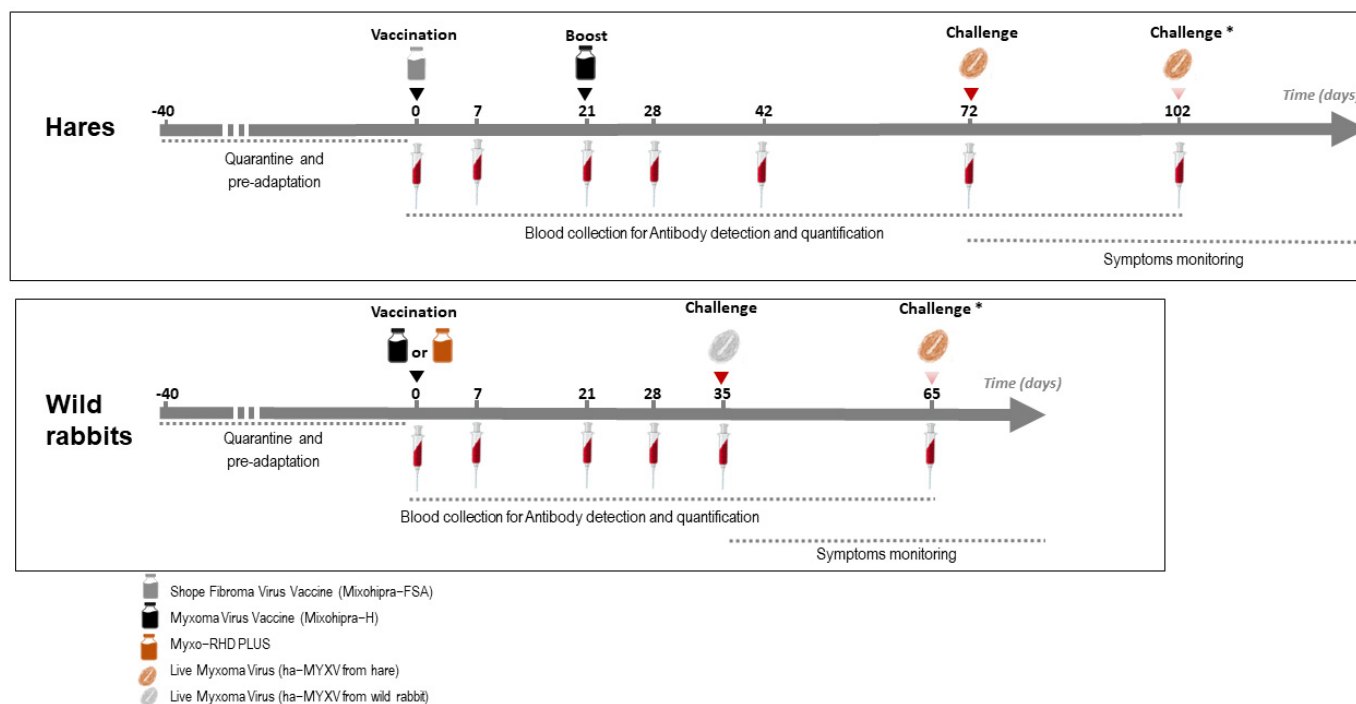
Figure 1. Schematic representation of the vaccination and sampling schedule performed on hares and rabbits (Studies 1 and 2). * The second challenge was only carried out on part of the animals (see Section 2.8).

2.4. Sampling and Blood Analyses

Given the nervous natural behaviour of wild leporids, wilder in the Iberian hare than in the wild rabbit, there is a high risk of self-injury during handling, especially affecting the lumbar spine following sudden movements in reaction to human presence or restraint. For this reason, the collection of samples was minimized to avoid the impact of stress on the results and to reduce the risk of harming the animals during the procedures.

The animals were handled with slow but assertive movements without sedation or anaesthesia. Blood collections were performed according to the method described by [13], without midazolam administration to avoid the effects of recurrent sedation.

The volume of blood collected from hares and rabbits was 6 mL and 1 mL, respectively. The blood was divided into three collection tubes, namely dry for serology, EDTA for hemogram and lithium heparin for biochemical analyses.

On the same day of sampling, serum was separated from the clot by centrifugation at $1000\times g$ for 10 min at 4 °C. The haematological analyses were performed automatically using the Procyte Dx haematological counter (IDEXX®) with further manual correction. The haematocrit was confirmed and corrected by the evaluation of the microhematocrit tube.

The relative count of leukocytes and the search for haemoparasites were performed by microscopical examination (400–1000× magnification) of blood and buffy coat smears stained with Diff-Quik.

Biochemical analyses were performed in a Catalyst One Chemistry Analyzer (IDEXX, Westbrook, ME, USA) using the Chem 15 CLIP consumables. All the analyses were performed in duplicate.

2.5. Serological Analyses

Sera were analysed for MYXV antibodies using a commercial indirect ELISA (iELISA) (Civtest® Cuni Mixomatosis, Hipra, Girona, Spain), following the manufacturer's instructions. Positive and negative controls (rabbit sera, provided in the kit) and samples were tested in duplicate. Results were expressed as Relative Index 10 (RI10).

All sera were also analysed by an in house immunofluorescence test (IFT) [7] using the intervals previously described [12].

The titre of the immunofluorescence test was estimated using 4 replicates for each dilution. Some hare sera were additionally tested at the OIE International Reference Laboratory of Myxomatosis (IZLER, Brescia, Italy), using a competitive ELISA (cELISA).

The titre of the seroneutralization test was estimated using 2 replicates for each dilution (method available upon request). Briefly, sera were initially inactivated by incubation at 56 °C for 30 min. Sera were then two-fold diluted from 1/4 to 1/2048 and incubated 2 h at 37 °C with 100 ffu of ha-MYXV (strain 20545PT20) using a 96-well plate. Then, 0.01×10^6 RK13 cells were sown in Gibco MEM medium (Thermo Fisher Scientific, Waltham, MA, USA) with 5% heat-inactivated foetal bovine serum—FBS (Thermo Fisher Scientific, Waltham, MA, USA). Virus plaques were visualized after 5 days of incubation and the titre considered the mean of the last dilutions that inactivated 100% of the virus.

2.6. Viruses

Viral isolation of ha-MYXV from eyelid samples from an infected wild rabbit (20545PT20, found dead in July 2020, Portugal) and from an infected hare (38455PT18, found dead in November 2018 in Portugal) were carried out using Rabbit Kidney (RK13) cells (ATCC-CCL-37). The rabbit and hare ha-MYXV strains were adapted to cell culture by seven passages, and then purified and titrated.

In detail, tissue samples were homogenised at 20% (*w/v*) in PBS containing penicillin, streptomycin and amphotericin B (antibiotic-antimycotic), used according to the manufacturer (Gibco, Waltham, MA, USA). Following centrifugation ($3000\times g$, 10 min, 4 °C), the supernatant was filtered through a 0.45-µm-pore-size filter (Millipore Express, Darmstad, Germany) and used to inoculate subconfluent (70%) RK13 cells grown in Eagle's medium supplemented with 5% FBS, penicillin, streptomycin and amphotericin B (antibiotic-antimycotic used at 1:100, Gibco) and 50 µg/mL gentamicin (Gibco). Cells were maintained at 37 °C in a humidified atmosphere with 5% CO2 and observed daily for cytopathic effect (CPE) by phase-contrast microscopy. Virus isolation was confirmed by an in-house IFT using MYXV positive hare serum [7]. Virus isolates were named 20545PT20 and 38455PT18, according to the identification of origin, and used for the immunofluorescence tests and seroneutralization assays.

Unlike the virus used in the IFT, produced as described above, the viruses used in challenge experiments were not subjected to cell culture passages to avoid mutations and recombination events during in vitro culturing, and correspond to the same isolates (20545PT20 and 38455PT18). The oedematous eyelids removed from these animals were washed seven times with sterile PBS pH 7.2 with mechanical agitation, to remove external tissue contaminants resulting from the accumulation of purulent material during the illness. After initial scalpel blade fragmentation, a 10% (*w/v*) tissue homogenate was prepared in sterile PBS pH 7.2, by mechanical maceration with 0.5 mm zirconia beads using four cycles of 15 s at 3000 rpm with an interval of 10 s (Precellys® Evolution). Before and after the process, the macerates were kept on ice.

After this initial process, the viruses were purified by centrifugation using a 36% sucrose cushion and then using a 24–40% sucrose step gradient [14]. The dilutions of the virus stock for the challenge were performed using sterile PBS pH 7.2. The final batch (corresponding to the dilution inoculated into the animals) was filtered using 0.45 μm pore filters and a sample submitted to incubation in blood agar, TSA-Tryptic Soy Agar and Sahoraud's dextrose agar, targeted to fungal agents according to routine methodology, to confirm the absence of contamination.

For stock titration, the virus was diluted in MEM (10-1 to 10-9) and adsorbed to 70% confluent RK13 cells, for 1 h at 37 °C. After 5 days of incubation, plates were washed with PBS and fixed with 70% Acetone (Scharlab, Barcelone, Spain), for 15 min at room temperature. Then, the wells were washed with PBS and stained with 0.4% crystal violet (Sigma-Aldrich, St. Louis, MO, USA), and visualized using an inverted microscope. The titre was estimated using 4 replicates for each dilution according to a published method [15]. The ha-MYXV stocks were then diluted in sterile PBS pH 7.2 to a concentration of 100 ffu/mL (focus-forming units per millilitre).

A similar method was used to titrate the vaccine stock (only for Mixohipra vaccines), using 100 μL of reconstituted vaccine to infect 70% confluent RK13 cells in 48-well plates. The reconstituted vaccine was 10-fold diluted using MEM until 10^{-6}. The vaccine virus was adsorbed for 1 h at 37 °C, and the titre was calculated after 5 days of incubation according to the previously described method [15].

2.7. Virus Detection by qPCR

Detection of ha-MYXV DNA by qPCR was used to confirm the isolation in RK13 cells [16]. The same molecular method was used to investigate the presence of the virus in faeces of the animals after vaccination and challenge, in tissues from the animals that died during the experiment, as well as in the drinking water. The presence of LeHV-5 was also analysed [4,17].

For nucleic acid extraction, cell supernatants or water samples were used directly for extraction, without dilution. Faeces or fresh samples of liver and spleen, kidney, lung, eyelid and genitalia were homogenised at 20% (*w/v*) with PBS and clarified at 3000 g for 5 min at 4 °C. Total DNA and RNA were extracted from 200 μL of the clarified supernatants, using the MagAttract 96 cador Pathogen Kit (Qiagen, Hilden, Germany) in a BioSprint 96 nucleic acid extractor (Qiagen, Hilden, Germany), according to the manufacturer's protocol.

Amplification reactions were performed in a Bio-Rad CFX96™ Thermal Cycler (Bio-Rad Laboratories Srl, Redmond, WA, USA), using the Multiplex PCR NZYTaq 2× Colourless Master Mix (NZYTech, Lisbon, Portugal).

2.8. Challenge

Hares (groups H-G1, HG-2, HG-3 and H-G4) were challenged at day 72 with the isolate ha-MYXV 38455PT18. Challenge of hares was carried out by inoculating 1 mL of the virus suspension subcutaneously (100 ffu/mL of ha-MXYV, diluted in sterile PBS pH 7.2), corresponding to the maximum estimated viral load delivered by arthropod vectors in nature [18]. In case of failure to develop disease and humoral response, hares were re-

inoculated with 1 mL of 1000 ffu/mL (ha-MXYV, isolate 38455PT18) at day 102 (Figure 1). Hares of study 3 were not submitted to challenge.

Wild rabbits (half of group R-G1 and groups R-G2 and R-G3) were challenged at day 35 with the isolate ha-MYXV 20545PT20 (Figure 1). Two of the negative controls (from R-G1, #000 and #001) were kept separately and were not inoculated. Thirty days later (day 65), these two rabbits were inoculated with the isolate ha-MYXV 38455PT18, the same used in the hare's assay. The rabbits challenge was performed with 1 mL of a 1000 ffu/mLdiluted in sterile PBS pH 7.2, inoculated subcutaneously, given the failure to induce disease in two hares when inoculated with 1 mL of a 100 ffu/mL virus dilution, as discussed below.

2.9. Clinical Signs Monitoring

During the vaccination trial and after challenge, the animals were continuously monitored by cameras, using black light for night vision. Daily visits to the installations by a veterinarian complemented surveillance. The cages allowed us to observe the animals without handling and to photograph the evolution of clinical signs. The cages also allowed us to touch the animals without the need to hold them, allowing us to assess the health and nutritional status as well as the presence of inflammatory lesions, for example at the site of vaccine inoculation, without disturbing the animals.

To follow the evolution of clinical signs after challenge, the animals' eyes were photographed daily. The hares' genitals were not monitored daily to minimize stress and risk of injuries, given the need to handle the animals. However, the daily monitoring of genitals in rabbits was carried out given their easier behaviour and due to the cage characteristics.

Sequential photographs of eyelid oedema were evaluated for each animal and subjectively classified (very mild, mild, moderate and marked). The palpebral fissure height was estimated and divided into four different categories (<25%, 25–50%, 50–75% and >75%).

2.10. Necropsy and Histopathology

Necropsy was performed according to routine procedures, and samples were collected for bacteriology (liver, spleen and lung), parasitology (gastrointestinal tract and liver), histopathology (lung, liver, spleen, kidney, eyelid and genitalia) and virology (liver, spleen, lung, kidney, eyelid, lip, urine, seminal vesicle, brain, bone marrow, spinal cord and genitalia) analyses, following the routine procedures. For histopathology, the fragments were fixated in 10% neutral buffered formalin (*w/v*), routinely paraffin-embedded, sectioned at 4 μm, and stained with Hematoxylin and Eosin (H&E).

2.11. Ethical and Legal Framework

The study was carried out in line with the measures identified in the National Plan for the control of rabbit haemorrhagic disease 2 (Dispatch 4757/2017, 31 May), operated through Project +Coelho 2, and approved by the National Authority for Animal Health (DGAV, Nr 79/ECVPT/20145) according to the National legislation (Decree-Law No. 113/2013, 7 August) after a positive declaration from the independent Advisory Body Responsible for Animal Welfare (ORBEA—INIAV, I.P.).

The Iberian hare specimens used in the experiment were from a pilot genetic reserve, established in 2019 within the scope of the +Coelho 2 Project, approved by ICNF. The Iberian hare founder population was captured in the field through events authorized by ICNF for the current proposal. The 14 hares selected for the study constitute the first generation born in captivity. The seropositive hare (#10) was captured in the field in October 2019. All wild rabbits used in this study were purchased from a certified wild rabbit captivity centre complying with the Portuguese legislation. Vaccination and challenge were conducted in a BSL-2 mobile unit belonging to INIAV I.P.

Taking into account the 3R policy, all steps of this assay that could be performed in vitro were maximized (replacement) (e.g., isolation, virus multiplication), reducing the number of animals to the minimum likely to give results for a pilot study (reduction) and all manipulation techniques, data analysis and maintenance of the animals, were thought

and designed specifically (refinement) for the Iberian hare and wild rabbit (e.g., BSL-2 built specifically for the study, specific cages were built and adapted to the needs of these two species. To minimize stress and self-inflicted damage, the animals were always handled by the same people and kept in a calm environment, etc.)

3. Results

The data described below refer to the quarantine and vaccination periods (from day −40 to day 72 in the hare trial and until day 35 in the rabbits' trial), i.e., before the challenge. Surveillance through day and night viewing cameras revealed an adequate adaptation to cages, expression of natural behaviour (Figure 2) and maintenance of water and food ingestion within the normal range.
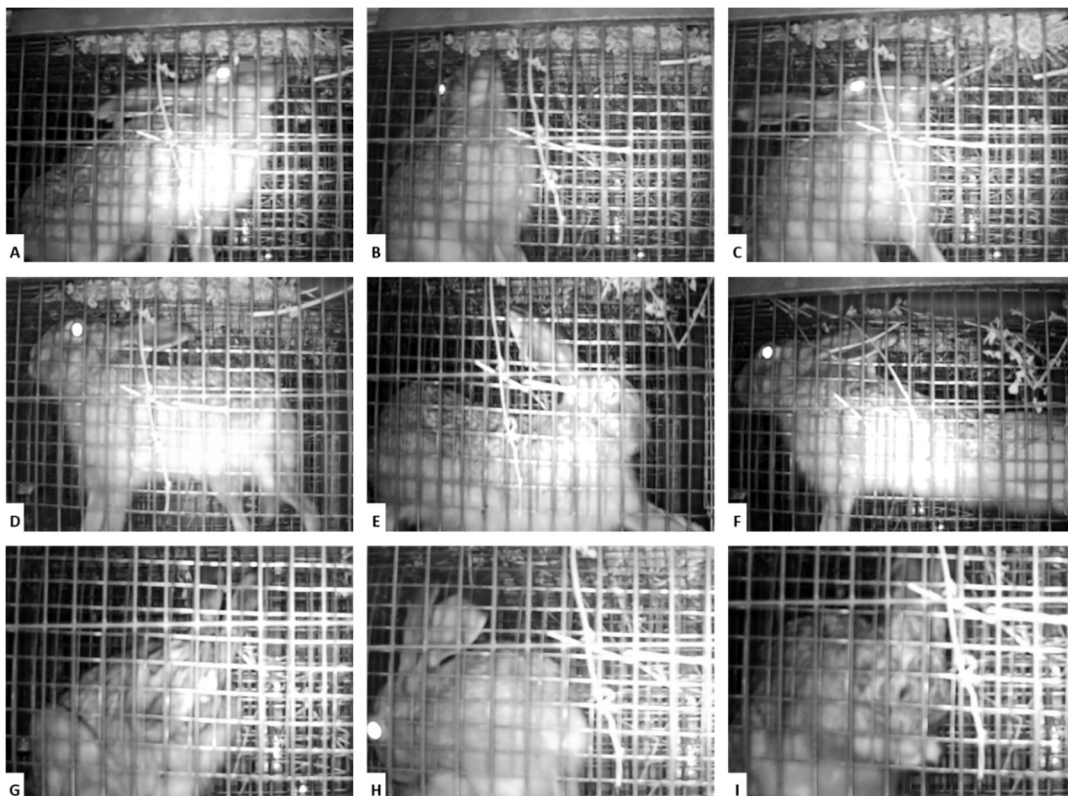
Figure 2. Pictures from the continuous monitoring performed by backlight cameras. The cages were designed to provide cognitive enrichment, making the access to alfalfa hay difficult (**A**–**C**), to allow all the hare's natural positions, namely muscle stretching (**D**–**F**). Animal welfare can be observed, among other factors, by the presence of grooming, which is very common in this species (**G**–**I**).

The 10 hares entered study 1 with an average weight of 1.90 ± 0.26 kg. All weight measurements were carried out in the morning, at approximately the same time. The maximum loss of weight before the challenge was 5.55%. Behaviour was stable throughout the entire experiment, with only a slight deterioration over the number of handlings, starting with an average rating of 1.3 in the first sampling and ending with 2.1 in the last one (after seven sampling procedures). The average respiratory frequency after the initial containment was 87 ± 13 breaths per minute (bpm) at the moment of first sampling and of 108 ± 9 bpm at the moment of the last sampling. The temperature was also measured in all sampling moments, showing an average of 39.5 ± 0.4 °C.

The rabbits did not have any remarkable loss in weight; on the contrary, they gained weight throughout the experiment (an average of 10.3%), the mean weight before the challenge being 1.12 ± 0.27 kg. The rabbits had an average temperature of 39.05 ± 0.1 °C in the five moments of blood sampling and a mean breath rate of 58 ± 23 bpm. The behaviour

of rabbits was substantially more favourable compared to hares, starting with an average rating of 1 in the first sampling and ending with 1.2 in the last (after five sampling events). In both species, the variations in weight, respiratory rate and rectal temperature were considered physiological, since the start of the study until the start of the challenge.

3.1. Humoral Immune Response to Vaccination

In general, the hares vaccinated with the lower dose of the two Mixohipra vaccines (H-G2), did not produce a serological response, except for hare #077, which achieved an RI10 title of 2.81. However, this hare died suddenly on day 51, hampering the follow-up of the next phases of the experiment. Necropsy showed that the cause of death of hare #077 was dysbiosis, probably due to stress, the animal being in good body condition.

Two of the three hares vaccinated with the higher dose of the two Mixohipra vaccines (H-G3), seroconverted, achieving an RI10 value of 5.8 (hare #043) and 7.1 (hare #044) by day 72 of the trial. Hare #042 showed no seroconversion during the experiment. The increase in serological response was higher after inoculation with the Mixohipra-FSA vaccine, namely between day 0 to day 21. The increase in serological response after boost vaccination with Mixohipra-H vaccine, between day 21 and day 42, was much lower or even absent (Table 1). By day 21, hares #043 and #044 registered a titre of 10 and 20, respectively, in the cELISA, while all the other hares remained negative. These titres remained unchanged, even after the boost vaccination.

These results were further confirmed in study 3, with all the five hares seroconverting. At day 0, the RI10 was < 1.0 in the five animals and 21 days later was 4.6, 4.9, 3.7, 8.2 and 4.9 for hares #013, #014, #020, #021 and #025, respectively. Twenty-one days after the second vaccination, the RI10 values obtained with the iELISA were 6.7, 6.6, 7.5, 11.2 and 5.4, respectively. Contrary to animals #043 and #044 (H-G3), which were boosted with Mixohipra-H, the administration of a boost with Mixohipra-FSA had a positive effect on the antibody titres.

As expected, the animals from the unvaccinated control group (H-G1) did not seroconvert (Table 1).

Rabbits #448, #446, and #447, vaccinated with Myxo-RHD PLUS (R-G3) seroconverted and on day 35 achieved an RI10 titre of 2.1, 4.8, and 5.4, respectively (Table 2). Rabbits #444, #445, and #442, vaccinated with Mixohipra-H (R-G2) seroconverted and on day 35 achieved an RI10 titre of 1.72, 2.15 and 4.11, respectively. As expected, none of the animals from the non-vaccinated group, R-G1 (#449, # 451, #000 and #001) developed anti-MYXV antibodies.

Table 1. Serological responses of hares (study 1) after vaccination and before challenge. Data were measured by indirect ELISA (RI10), immunofluorescence titration (IFT = titre in log2) and seroneutralization (SNT = titre).

Group	Hare ID	Vaccine	Technique	Day 0	Day 7	Day 21	Vaccine	Day 28	Day 42	Day 72
										Humoral Response
H-G1	#231	Not vac	iELISA	0.5	0.23	0.16	Not vac	0.13	0.19	0.23
			IFT	<2	<2	<2		<2	<2	<2
			SNT	<1/4	<1/4	<1/4		<1/4	<1/4	<1/4
	#232	Not vac	iELISA	0.35	0.16	0.39	Not vac	0.29	0.15	0.28
			IFT	<2	<2	<2		<2	<2	<2
			SNT	<1/4	<1/4	<1/4		<1/4	<1/4	<1/4
	#233	Not vac	iELISA	0.63	0.47	0.42	Not vac	0.22	0.22	0.08
			IFT	<2	<2	<2		<2	<2	<2
			SNT	<1/4	<1/4	<1/4		<1/4	<1/4	<1/4

Table 1. *Cont.*

Group	Hare ID	Vaccine	Technique	Day 0	Day 7	Day 21	Vaccine	Day 28	Day 42	Day 72
							Humoral Response			
H-G2	#076	1× M-FSA	iELISA	0.9	1.01	0.84	1× M-H	0.59	0.59	0.18
			IFT	<2	3	3.5		4	4	4
			SNT	<1/4	<1/4	<1/4		<1/4	<1/4	<1/4
	#077	1× M-FSA	iELISA	0.31	0.81	1.55	1× M-H	2.45	2.81	-
			IFT	<2	<2	4		4	5.5	
			SNT	<1/4	<1/4	<1/4		1/8	1/8	
	#078	1× M-FSA	iELISA	0.96	0.49	0.4	1× M-H	0.36	0.36	0.12
			IFT	<2	<2	3		3	3	3
			SNT	<1/4	<1/4	<1/4		<1/4	<1/4	<1/4
H-G3	#042	10× M-FSA	iELISA	0.6	0.36	0.25	10× M-H	0.24	0.18	0.03
			IFT	<2	<2	3		3	3	3
			SNT	<1/4	<1/4	<1/4		<1/4	<1/4	<1/4
	#043	10× M-FSA	iELISA	0.79	0.62	5.55	10× M-H	5.05	5.5	5.8
			IFT	<2	4.5	6.5		7	7	7.5
			SNT	<1/4	<1/4	1/16		1/16–1/32	1/16–1/32	1/16–1/32
	#044	10× M-FSA	iELISA	0.75	0.36	5.57	10× M-H	6.87	6.7	7.1
			IFT	<2	3.5	7		7.5	7	8.5
			SNT	<1/4	<1/4	1/16		1/16–1/32	1/16–1/32	1/16–1/32
H-G4	#10	Natural immunity	iELISA			7.09	Natural immunity	7.1	7.19	7.28
			IFT			9		9	9	9
			SNT			1/128		1/128	1/128	1/128

1× M-FSA, vaccinated with 2.90 × 10^4 ffu of Mixohipra-FSA; 10× M-FSA, vaccinated with 2.90 × 10^5 ffu dose of Mixohipra-FSA; 1× M-H, vaccinated with 1.95 × 10^4 ffu dose of Mixohipra-H; 10× M-H, vaccinated with 1.95 × 10^5 ffu dose of Mixohipra-H; Not vac, not vaccinated.

Table 2. Serological responses of rabbits (study 2) after vaccination and before challenge. Data were measured by indirect ELISA (RI10), immunofluorescence titration (IFT = titre in log2) and seroneutralization (SNT = titre). RI10.

Group	Rabbits ID	Vaccine	Technique	Day 0	Day 7	Day 21	Day 28	Day 35
				Humoral Response				
R-G1	#449	Not vac	iELISA	0.05	0.06	0.07	0.06	0.02
			IFT	<2	<2	<2	<2	<2
			SNT	<1/4	<1/4	<1/4	<1/4	<1/4
	#451	Not vac	iELISA	0.22	0.01	0.1	0.27	0.2
			IFT	<2	<2	<2	<2	<2
			SNT	<1/4	<1/4	<1/4	<1/4	<1/4
	#000	Not vac	iELISA	0.13	0.09	0.15	0.18	0.31
			IFT	<2	<2	<2	<2	<2
			SNT	<1/4	<1/4	<1/4	<1/4	<1/4
	#001	Not vac	iELISA	0.32	0.27	0.38	0.21	0.14
			IFT	<2	<2	<2	<2	<2
			SNT	<1/4	<1/4	<1/4	<1/4	<1/4
R-G2	#442	1× M-H	iELISA	0.01	0.05	1.79	2.33	4.11
			IFT	<2	4	6	7.5	9
			SNT	<1/4	<1/4	<1/4	1/8	1/32
	#444	1× M-H	iELISA	0.04	0.18	1.1	1.04	1.72
			IFT	<2	2	5.5	6	7
			SNT	<1/4	<1/4	<1/4	1/4	1/8
	#445	1× M-H	iELISA	0.1	0.29	0.83	1.04	2.15
			IFT	<2	3	<2	6	8.5
			SNT	<1/4	<1/4	<1/4	1/4	1/8

Table 2. *Cont.*

Group	Rabbits ID	Vaccine	Technique	Day 0	Day 7	Day 21	Day 28	Day 35
						Humoral Response		
R-G3	#446	1× M-RHD	iELISA	0.31	0.36	2.3	3.05	4.78
			IFT	<2	3.5	6.5	8	9
			SNT	<1/4	<1/4	1/8	1/16	1/64
	#447	1× M-RHD	iELISA	0.35	0.21	5.32	5.42	5.39
			IFT	<2	4.5	9.5	10	10
			SNT	<1/4	1/4	1/128	1/128	1/128
	#448	1× M-RHD	iELISA	0.26	0.51	1.21	1.09	2.15
			IFT	<2	4	5.5	6	7
			SNT	<1/4	<1/4	1/4	1/8	1/8–1/16

1× M-H—vaccinated with 1.95 × 10^4 ffu dose of Mixohipra-H; 1×-M-RHD—vaccinated with 1 dose of Myxo RHD-PLUS; Not vac—not vaccinated.

3.2. Hematologic and Biochemical Analyses of Blood Samples Obtained during Vaccination

Monitoring of the different parameters analysed in the hemogram (RBC, HCT, HGB, MCV, MCH, MCHC, RDW, reticulocytes, WBC, neutrophils, lymphocytes, monocytes, eosinophils, basophils, platelets, MPV, PDW, PCT) showed no remarkable changes after vaccination in any of the hares and rabbits. Likewise, regarding the biochemistry analyses, no remarkable variations in glucose, creatinine, blood urea nitrogen, ALT, ALKP or GGT values were observed. Interestingly, the albumin/globulins ratio, in the case of hare #043 and hare #044 (both from H-G3), showed a decrease between days 21 and 28 after the first vaccination, coinciding with the seroconversion.

By day 30 after the challenge, sick hares showed lower haematocrit of 35.33 ± 4.51% (compared to 53.44 ± 3.24% in the healthy animals) and a leucocytosis of 18.82 ± 4.86 K/μL (WBC) (compared to 5.65 ± 1.54 K/μL (WBC) in the healthy hares), mainly resulting from a neutrophilia of 15.30 ± 7.32 K/μL (compared to 2.53 ± 1.33 K/μL in the healthy hares).

3.3. Clinical Course after Challenge

Hares #233 (H-G1) and #042 (H-G3) did not develop any signs of disease (Table 3) after the first virus inoculation of 1 mL (100 ffu/mL, subcutaneously), and no virus was detected in the blood, stool or conjunctival swab on day 15. Additionally, these hares did not seroconvert and were inoculated a second time, 30 days after the first virus challenge, with the same isolate, but a 10-fold higher dose (1 mL of 1000 ffu/mL). Both died after developing signs of myxomatosis (Table 3).

The average incubation period of myxomatosis in hares was 11.3 ± 2.6 days considering the 8 hares, and 11.8 ± 2.2 excluding the two seropositive hares (#043 and #044), taking as a clinical reference the alterations developed in the eyelids (Table 3 and Figure 3). The evolution of oedema in the genitals was not monitored, given the difficulty of handling the sick animals and the stress effect of such handling. From the onset of symptoms, it took an average of 17.8 ± 8.5 days until death occurred or euthanasia was carried out for animal welfare purposes. Hares #043 and #044 recovered totally, respectively, in 13 and 19 days after the challenge. Hares of study 3 were not submitted to challenge.

Figure 3. Clinical signs of myxomatosis of four hares involved in the study. Hares #231 and #232 (H-G1) were not vaccinated. Hares #043 and #044 belonged to the H-G3 group (vaccinated with 2.90×10^5 ffu dose of Mixohipra-FSA). D-day after challenge.

Hares #233 (from H-G1, not vaccinated), #078 (from H-G2, vaccinated with low dose of Mixohipra-FSA and Mixohipra-H) and #042 (from H-G3, vaccinated with a high dose of Mixohipra-FSA and Mixohipra-H but challenged twice) developed severe myxomatosis and died. These hares, like those euthanized after developing severe myxomatosis (hares #231 and #232 not vaccinated (H-G1) and hare #076 vaccinated with low dose (H-G2)) lost on average 483.3 ± 147.2 g of weight from the day of virus challenge to the day of death (Table 3). In contrast, hares that developed light and shorter forms of the disease (hares #043 and #044 vaccinated with high dose (H-G3)) lost at the most 116.7 ± 28.9 g (Table 3). Comparing the hares that became very sick (>50% eyelid closed) with healthy ones, food intake decreased from 170–200 g of oats per day to 40–50% less at maximum. The opposite happened with water intake that rose from 50–70 mL to 80–110 mL per day at maximum.

The average incubation period of myxomatosis in positive control rabbits (R-G1, not vaccinated) was 8 days for the rabbits (#449 and #451) inoculated with isolate 20545PT20 (isolated from a wild rabbit) and 8 days for the rabbits #000 and #001 inoculated with isolate 38455PT18 (isolated from an Iberian hare) considering the alterations in the eyelids (oedema and eye closure) as reference (Table 4 and Figure 4), corresponding to an average of 6.3 ± 2.1 days in the four rabbits.
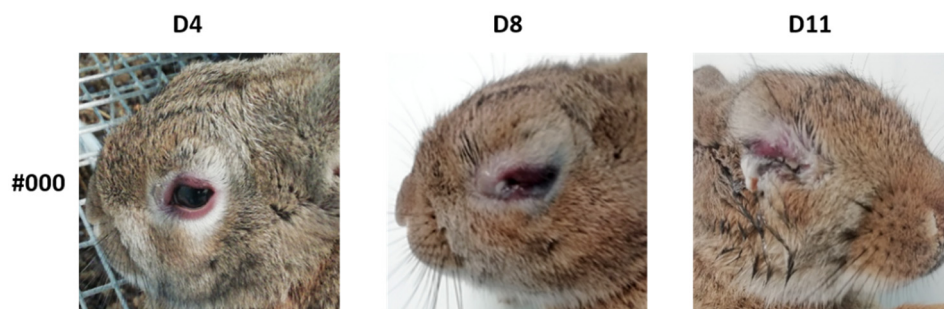
Figure 4. The clinical course of rabbit #000 (non-vaccinated, belonging to group R-G1) inoculated with virus isolated from Iberian hare (1 mL of 1000 ffu/mL, 38455PT18). D-day after challenge. Note very mild (D4), moderate (D8) and (D11) marked eyelid oedema.

In rabbits, it took an average of 5.25 ± 1.5 days from the onset of symptoms until death occurred, and an average of 11.5 ± 0.56 days from the virus challenge until death, with an average weight loss of 73.5 ± 45.0 g. None of the vaccinated rabbits showed symptoms of myxomatosis (Table 4).

Interestingly, the incubation period among rabbits inoculated with wild rabbit virus isolate (20545PT20) was longer (8 days) compared to Iberian hare virus isolate (38455PT20) (4.5 days). After the onset of symptoms, rabbits inoculated with strain 20545PT20 died within 4 days and rabbits inoculated with a strain of Iberian hare died within 6.5 days. As expected, rabbits with a longer course of disease showed more severe pathological signs (Table 4).

The MYXV antibodies estimated by iELISA at the death or recovery moments revealed RI10 values of 9.12, 1.82 and 0.23 for the #231, #232, #233 (H-G1, nonvaccinated); 2.62 and 0.12 for #076, #078 (H-G2, vaccinated with the lower dose); and 0.23, 31.00 and 28.90 for #042, #043 and #044 (H-G3, vaccinated with the higher dose), respectively.

Hare #010 (H-G4) did not show seroconversion after the first challenge with 100 ffu, but had a small increase in RI10 after the second inoculation to 13.32. LeHV-5 was neither detected in blood cells nor skin samples, suggesting the absence of latency or active replication in any of the hares, ruling out the LeHV-5 contribution to the clinical picture and immune response.

In the rabbits, he MYXV antibodies estimated by iELISA at the death (for R-G1) or fifteen days after challenge (R-G2 and R-G3) revealed RI10 lower than 2.0 for the #449, #451, #000 and #001 (R-G1, nonvaccinated); 21.79, 4.31 and 5.71 for #442, #444, and #445 (R-G2, vaccinated with Mixohipra-H); and 33.02, 23.44, 24.48 for #446, #447 and #448 (R-G3, vaccinated with Myxo-RHD PLUS), respectively.

3.4. Virus Presence in Drinking Water and Faeces

During the two vaccination trials, no MYXV-DNA was detectable in the drinking water or the hares' faeces prior challenge.

MYXV-DNA was detected in faeces of hare #231 (H-G1), seven days after virus challenge, four days before the first signs of disease were noticed. For the remaining hares, virus shedding coincided with the appearance of the first clinical signs or appeared 3 to 4 days later, around day 15 or 16 after the virus challenge. The maximum viral load found on faeces was 1.10×10^9 DNA copies/mg and the average value was 4.00×10^7 DNA copies/mg, considering only the DNA-positive samples. The virus was no longer detected in faeces after the animals recovered clinically (clinical signs disappeared) or, in some cases, 48 h before the disappearance of signs of disease.

Table 3. Clinical data of Iberian hares after challenge with ha-MYXV (38455PT18).

Group	Animal ID	0	1–9	10–15	16–20	21–25	26–30	31–40	D41–D50
						Days after Virus Challenge			
H-G1	Hare #231 Not vac	Virus challenge with 1 mL (100 ffu/mL) of ha-MYXV (38455PT18)	N	D11-very mild eyelid oedema. <25% of eye closure. D13—mild eyelid oedema. <25% of eye closure. D14—moderate eyelid oedema. 50% of eye closure.	D16 marked eyelid oedema. 50–75% of eye closure.	D25 marked eyelid oedema. >75% of eye closure.	D30-marked eyelid and anogenital oedema. 100% of eye closure. Anorexia and dyspnea. Euthanasia.		
	Hare #232 Not vac		N	D12—mild eyelid oedema. <25% of eye closure. D14—moderate eyelid oedema. 25–50% of eye closure.	D18 marked eyelid oedema. 50–75% of eye closure.	D25 marked eyelid oedema. >75% of eye closure.	D30 marked eyelid and anogenital oedema. 100% of eye closure. Anorexia and dyspnea. Euthanasia.		
	Hare #233 Not vac		N	N	N	N	D30—Second virus challenge with 1000 ffu of ha-MXYV (38455PT18), day 102 in Figure 1.	D40—very mild eyelid oedema. <25% of eye closure. D42—mild eyelid oedema. <25% of eye closure.	D45—Marked eyelid, foreskin and anogenital oedema. 50–75% of eye closure. Anorexia. D50-Death
H-G2	Hare #076 1× M-FSA 1× M-H		N		D16—very mild eyelid oedema. <25% of eye closure. D18—very mild eyelid oedema. <25% of eye closure.	D20—mild eyelid oedema. <25% of eye closure.	D20—moderate eyelid oedema. 25–50% of eye closure. D30—marked eyelid oedema. 50–75% of eye closure.	D40—marked eyelid oedema. >75% of eye closure.	D48—marked eyelid and foreskin oedema. >75% of eye closure. Anorexia and dyspnoea. Euthanasia.
	#078 Hare 1× M-FSA 1× M-H		N	D12—very mild eyelid oedema. <25% of eye closure.	D16—mild eyelid oedema. <25% of eye closure.	D20—mild eyelid oedema. <25% of eye closure. Death			

Table 3. *Cont.*

Group	Animal ID	0	1–9	10–15	16–20	21–25	26–30	31–40	D41–D50
									Days after Virus Challenge
H-G3	Hare #042 10× M-FSA 10× M-H		N	N	N	N	D30—Second virus challenge with 1000 ffu of ha-MXYV (38455PT18), day 102 in Figure 1.	D40—very mild eyelid oedema. <25% of eye closure. D45—mild eyelid oedema. <25% of eye closure.	D50—mild eyelid oedema. <25% of eye closure D60—moderate eyelid, testis and foreskin oedema. 25–50% of eye closure. Anorexia. Death.
	Hare #043 10× M-FSA 10× M-H		N	D12-very mild eyelid oedema. <25% of eye closure.	D16—Left l ower eyelid erythema.	D25—N	N	N	N
	Hare #044 10× M-FSA 10× M-H		D7—mild eyelid oedema. <25% of eye closure.	D12—mild eyelid oedema. <25% of eye closure.	D14-D20—moderate eyelid oedema. 25–50% of eye closure.	D21—Beginning of crusting and improvement of eye opening.	D26—N, in addition to scars on the eyelids.		
H-G4	Hare #10 Natural immunity		N	N	N	N	D30-Second virus challenge with 1000 ffu of 38455PT18.	N	N

1× M-FSA,—vaccinated with 2.90×10^4 ffu dose of Mixohipra-FSA; 10× M-FSA—vaccinated with 2.90×10^5 ffu dose of Mixohipra-FSA; 1× M-H—vaccinated with 1.95×10^4 ffu dose of Mixohipra-H; 10× M-H—vaccinated with 1.95×10^5 ffu dose of Mixohipra-H, Not vac-Not vaccinated; D-day, N—no disease signs.

Table 4. Clinical data of wild rabbits after challenge with ha-MYXV isolated from wild rabbit (20545PT20) or Iberian hare (38455PT18).

Group	Animal ID	0	1–9	10–15	16–20	21–25	26–30	31–40	D41–D50
				Days after Virus Challenge					
RG-1 Not vac	Rabbit #000	Virus challenge of 1000 ffu of ha-MYXV (38455PT18), day 65 in Figure 1.	D4—very mild eyelid oedema. No genital changes. D8—moderate eyelid and genital oedema. 50–75% of eye closure.	D11—Marked eyelid, foreskin and genital oedema. >75% of eye closure Anorexia. Death.					
	Rabbit #001		D5—very mild eyelid and genital oedema. D8—moderate eyelid and genital oedema. 50–75% of eye closure.	D11—Marked eyelid, foreskin and genital oedema. >75% of eye closure Anorexia. Death.					
	Rabbit #449		D8—very mild eyelid oedema. <25% of eye closure. No genital changes.	D12—Very mild eyelid and foreskin oedema. <25% of eye closure. No genital oedema. Death.					
	Rabbit #451		D8—very mild eyelid oedema. <25% of eye closure. No genital changes.	D12—Very mild eyelid and foreskin oedema. <25% of eye closure. No genital oedema Death.					
RG-2 1× M-H	Rabbit #442	Virus challenge of 1000 ffu of ha-MYXV (20545PT20), day 35 in Figure 1.	N	N	N	N	N	N	N
	Rabbit #444		N	N	N	N	N	N	N
	Rabbit #445		N	N	N	N	N	N	N
RG-3 1× M-RHD	Rabbit #446		N	N	N	N	N	N	N
	Rabbit #447		N	N	N	N	N	N	N
	Rabbit #448		N	N	N	N	N	N	N

1× M-H—vaccinated with 1.95×10^4 ffu dose of Mixohipra-H; 1× M-RHD—vaccinated with 1 dose of Myxo-RHD PLUS; Not vac-not vaccinated; D-day, N—no disease signs.

The type of drinking fountain used for hares consisted of a small shell-shaped reservoir containing a limited volume of water (about 5 mL), from which water was sampled. In hares that developed erosive lesions in the oral mucosa (#231, #232, #233, #042 and #076) the virus was detected in water samples by qPCR. The average viral load in the DNA-positive drinking water samples was 5.00×10^6 DNA copies/mL with a minimum of 6.32×10^3 and a maximum of 4.73×10^7 DNA copies/mL. For one drinking water sample (with a viral load of 1.30×10^7), it was possible to isolate the virus in RK13 cells.

The faeces analyses were not performed in the rabbit trial due to the characteristics of the cages that do not allow the separation of the faeces from each animal. Likewise, the rabbits' pacifier drinkers do not allow the analysis of the drinking water, and therefore this analysis was not performed.

3.5. Necropsy, Histopathology and Virus Loads in Tissues

The necropsy and histopathology data of hares from group H-G1 (#231, #232, #233), H-G2 (#076 and #078) and H-G3 (#042) revealed the expected lesions found in naturally infected animals by ha-MYXV [3,19,20] namely the oedema of eyelids and ano-genital, the production of myxoid tissue and the secondary bacterial infection. No remarkable histopathological changes were observed in animals #043 and #044 (H-G3) artificially immunized by the high dose vaccine, or in #10 (H-G4) naturally immunized.

Rabbits #449, #451, #000, #001 (Table 5) revealed the expected lesions found in naturally infected rabbits by the ha-MYXV [7,8], namely eyelid, ano-genital and alveolar oedemas.

Organs sampled but without description in Table 5 were considered without relevant changes. No myxoma lesions (also called "pseudotumours" or "tumour-like lesions") were observed in the skin of any animal (rabbit and hares) during the clinical course of the disease or after death, not even in the virus inoculation zone.

No pathological findings in necropsy and histopathology were found in hares #043 (H-G3, vaccinated with high dose) and #010 (H-G4, naturally immunized) and all the vaccinated rabbits: #442, #444 and #445 (R-G2, vaccinated with M-H) and #446, #447 and #448 (R-G3, vaccinated with Myxo-RHD PLUS), and they were also negative to bacteriological and parasitological analyses (not included in Table 5). Hare #044 (H-G3) only presented mild scar on the eyelids.

Several pathogenic bacteria were found in the animals included in this study (Table 5). In general, mild infections by *Eimeria* species were found in sick animals, and these parasites were very frequently also found in wild animals that died from myxomatosis or in healthy animals [20]. Despite the infection, no signs compatible with enteritis were detected at necropsy and no diarrhoea events were registered during the entire course of the trial in these animals.

The viral loads in the different tissues (Table 6) were determined by qPCR [16]. The highest viral loads were registered in the eyelids (mean of all positive hares of $1.10 \times 10^{10} \pm 1.02 \times 10^{10}$ DNA copies/mg tissue and mean of all positive rabbits of 4.79×10^{10} DNA copies/mg tissue), lips (mean of all positive hares of $9.30 \times 10^9 \pm 1.60 \times 10^{10}$ DNA copies/mg tissue and mean of all positive rabbits of $1.22 \times 10^9 \pm 8.88 \times 10^8$ DNA copies/mg tissue) and genitalia (mean of all positive hares of $1.65 \times 10^{10} \pm 2.52 \times 10^{10}$ DNA copies/mg tissue and mean of all positive rabbits of $2.53 \times 10^{10} \pm 2.43 \times 10^{10}$ DNA copies/mg tissue).

Overall, no virus was detected in vaccinated rabbits independently of the vaccine used, but was detected in all non-vaccinated rabbits. In all hares submitted to virus challenge, virus was detected in the tissues except for 2 out of the three hares vaccinated with $10\times$ M-FSA vaccine, which seroconverted.

Table 5. Necropsy, bacteriological and parasitological data from hares and rabbits.

Group	Animal ID	Challenge	Pathological Examination					Bacteriological Examination from Liver, Spleen and Lungs	Parasitological Examination
			Eyelid/Ano-Genitalia/Lip	Liver	Spleen	Lungs	Kidney/Other		
H-G1	Hare #231	100 ffu of ha-MYXV isolated from hare (38455PT18)	Macroscopic: Marked eyelid, lips, genitalia and anus oedema. Thickening and congestion of the scrotal sac wall with necrosis. Accumulation of fibrin within the scrotal pouches. Congestion of the testicles. Microscopic: Eyelid with necrosis of the epidermis and conjunctiva with bacterial infiltration. Scrotum with oedema at the dermo-epidermal junction with detachment of the epidermis. Lip with necropurulent dermatitis and presence of extensive bacterial clusters. Testis with absence of germ cells, persisting only Sertoli cells. Epididymis with necrosis of the lining epithelium and accumulation of necrotic cells in the lumen of the ducts. *Vas deferens* with accumulation of myxoid tissue underlying the lamina propria.	Moderate periportal infiltration by mononuclear cells	N	N	Kidney with perivascular lymphocytic infiltration. Empty stomach.	*Escherichia coli* and *Stenotrophomonas maltophilia*	Mild infection by *Eimeria* spp.
	Hare #232		Macroscopic: Marked eyelid, lips, genitalia and anus oedema. Microscopic: Eyelid with epidermal hyperplasia and extensive proliferation of myxoid tissue in depth to the conjunctiva. Lips with epidermal hyperplasia, ballooning degeneration of keratinocytes, intense production of myxoid tissue, infiltration of muscle tissue by mononuclear cells and heterophils. Prepuce with extensive and severe necropurulent dermatitis with strong bacterial infiltration, myxoid tissue in the dermis, diffuse infiltration by heterophils in depth, necropurulent foci.	Cellular infiltration, namely by heterophils in the porta spaces and occasionally in the sinusoid capillaries.	Marked depletion of lymphocytes.	Multifocal purulent alveolitis.	Kidney with perivascular infiltration by mononuclear cells. Empty stomach.	*Stenotrophomonas maltophilia, Staphylococcus equorum* and *Staphylococcus xylosus*	Mild infection by *Eimeria* spp.

Table 5. *Cont.*

Group	Animal ID	Challenge	Pathological Examination					Bacteriological Examination from Liver, Spleen and Lungs	Parasitological Examination
	Hare #233	1000 ffu of ha-MYXV isolated from hare (38455PT18)	Macroscopic: Nodular thickening on the eyelids and lips and oedema of the prepuce. Microscopic: Eyelid with epidermal hyperplasia and vacuolization of keratinocytes. Proliferation of myxoid tissue in the dermis. Diffuse microhaemorrhages and foci of infiltration by heterophils. Conjunctival epithelial hyperplasia and underlying stromal oedema. Lips with epithelial hyperplasia and marked vacuolization of keratinocytes with proliferation of myxoid tissue in the dermis and intense infiltration by heterophils. Foreskin with thick necropurulent exudate and extensive haemorrhages, presence of myxoid tissue and infiltration by heterophils.	N	Marked depletion of lymphocytes.	Lung collapse.	Empty stomach.	*Escherichia coli* and *Enterobacter sakazakii*	Mild infection by *Eimeria leporis*.
H-G2	Hare #076	100 ffu of ha-MYXV isolated from hare (38455PT18)	Macroscopic: Marked oedema of the prepuce, eyelids and lips. Empty stomach. Microscopic: Eyelid with dermal oedema. Lips with foci of epithelial hyperplasia and vacuolization of keratinocytes. Foreskin with marked oedema of the dermis.	Liver with periportal infiltration by mononuclear cells and heterophils in sinusoid capillaries.	Mild depletion of lymphocytes.	Congestion.		*Escherichia coli*	N
	Hare #078		Macroscopic: Marked oedema of the foreskin, eyelids and lips. Presence of small ulcers on the lips. Microscopic: Lips with foci of epithelial hyperplasia and vacuolization of keratinocytes. Foreskin with marked oedema of the dermis. Epididymis with oedema and extensive haemorrhages in the basement membrane zone. Hyperplasia of the duct lining epithelium and desquamation of the ductal epithelium. Infiltration by heterophils. Eyelid with dermal oedema.	Liver with periportal infiltration, mainly by mononuclear cells and heterophils in sinusoid capillaries.	Marked depletion of lymphocytes.	Congestion.	Kidney with the presence, mostly perivascular, of small clusters of lymphoid cells. Empty stomach.	*Enterococcus gallinarum*	Marked infection by *Eimeria* spp.

Table 5. *Cont.*

Group	Animal ID	Challenge	Pathological Examination					Bacteriological Examination from Liver, Spleen and Lungs	Parasitological Examination
H-G3	Hare #042	1000 ffu of ha-MYXV isolated from hare (38455PT18)	Macroscopic: Nodular thickening and moderate oedema and nodular thickening of eyelids, nose, lips and testis. Oedema of the extremities of the hind limbs. Microscopic: Eyelid with epidermal hyperplasia and vacuolization of keratinocytes. Moderate proliferation of myxoid tissue in the dermis. Lips with necropurulent lesions, moderate epithelial hyperplasia and vacuolization of keratinocytes with proliferation of myxoid tissue in the dermis and infiltration by heterophils. Foreskin with moderate presence of myxoid tissue and infiltration by heterophils.	Congestion.	Marked depletion of lymphocytes.	*Congestion.*	Empty stomach.	*Vibrio vulnificus*	N
	Hare #044	100 ffu of ha-MYXV isolated from hare (38455PT18)	Macroscopic: Mild scar on the eyelids.	N	N	N	N	N	N
R-G1	Rabbit #449 and Rabbit #451	1000 ffu of ha-MYXV isolated from rabbit (20545PT20)	Macroscopic: Very mild oedema of the eyelids and lips. Presence of extensive pleural effusion with fibrin. Microscopic: Eyelid with mild oedema of the deep dermis.	Congestion of Liver (#449).	Spleen with total necrosis of lymphoid follicles with only a very congested red pulp visible.	Lung congestion and oedema. Pleural efusion. Necrosis of peribronchial lymphoid tissue.	Full stomach.	N	N
	Rabbit #000 and Rabbit #001	1000 ffu of ha-MYXV isolated from hare (38455PT18)	Macroscopic: Marked swelling of the eyelids, lips and external genitalia. Presence of purulent mucous exudate on the eyelids. Reduced content in the stomach. Microscopic: Eyelid with marked oedema, small foci of myxoid tissue in the dermis, purulent conjunctivitis of bacterial etiology. Lip with oedema and small foci of myxoid tissue.	N	Spleen with congestion and necrosis of lymphoid follicles.	N	N	N	Medium infection with *Eimeria perforans*.

N—no remarkable changes or negative to bacterial or parasitological analysis.

Table 6. Viral loads in the hare and rabbit tissues after challenge.

ID	Vaccination Data		Viral Loads in Different Tissues (DNA Copies/mg Tissue)												
	1st	2nd	Liver and Spleen	Lung	Duodedum	Kidney	Eyelid	Lip	Genitalia	Urine	Seminal Vesicle	Faeces	Brain	Bone Marrow	Spinal Cord
Hares															
#231	Not vac	Not vac	1.60×10^7	1.47×10^8	5.76×10^6	1.71×10^7	2.88×10^9	1.75×10^9	2.23×10^7	7.44×10^6	1.78×10^9	3.98×10^8	4.05×10^6	1.31×10^6	1.53×10^6
#232	Not vac	Not vac	1.10×10^8	1.23×10^8	3.66×10^7	3.15×10^9	1.24×10^{10}	7.47×10^9	1.24×10^{10}	5.47×10^3	5.42×10^6	1.83×10^6	4.95×10^5	3.70×10^5	5.45×10^6
#233	Not vac	Not vac	6.73×10^8	6.28×10^8	4.03×10^6	4.39×10^8	1.34×10^{10}	3.88×10^9	5.33×10^{10}	NT	5.37×10^5	1.52×10^8	2.29×10^8	6.83×10^4	9.91×10^4
#076	1× M-FSA	1× M-H	2.55×10^5	2.33×10^5	5.59×10^4	1.85×10^6	1.34×10^7	1.11×10^6	2.08×10^8	2.16×10^5	ND	1.16×10^5	ND	3.11×10^3	ND
#077	1× M-FSA	1× M-H	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
			This animal was not submitted to challenge but was analysed to MYXV to eliminate as the cause of death												
#078	1× M-FSA	1× M-H	1.17×10^8	4.39×10^8	7.20×10^7	2.22×10^8	2.89×10^{10}	4.16×10^{10}	5.40×10^9	NT	1.36×10^5	1.50×10^9	6.26×10^6	3.22×10^4	8.54×10^6
#042	10× M-FSA	10× M-H	2.90×10^7	3.06×10^7	8.72×10^6	1.50×10^7	8.46×10^9	1.08×10^9	7.73×10^9	NT	NT	7.51×10^8	7.42×10^4	9.25×10^4	2.36×10^5
#043	10× M-FSA	10× M-H	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
#044	10× M-FSA	10× M-H	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
#10	Natural immunity		ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Rabbits															
#442	1× M-H		ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
#444			ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
#445			ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
#446			ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
#447	1× M-RHD		ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
#448			ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
#449	Not vac		2.30×10^{10}	2.03×10^{10}	7.16×10^8	9.97×10^8	1.29×10^{10}	1.36×10^9	4.19×10^{10}	2.84×10^7	ND	2.22×10^4	ND	1.02×10^2	ND
#451			1.24×10^{10}	3.48×10^{10}	1.04×10^8	4.94×10^9	1.87×10^{10}	9.97×10^8	3.01×10^9	8.72×10^6	NT	1.15×10^4	ND	8.91×10^2	ND
#000	Not vac		9.91×10^9	7.74×10^{10}	1.25×10^8	1.45×10^9	6.98×10^{10}	2.33×10^9	6.07×10^9	1.15×10^8	NT	1.24×10^5	3.13×10^4	ND	ND
#001			3.32×10^8	2.11×10^9	2.93×10^7	8.32×10^9	9.02×10^{10}	1.89×10^8	5.04×10^{10}	2.04×10^6	NT	2.25×10^3	4.12×10^3	2.15×10^0	ND

1× M-FSA—vaccinated with 2.90×10^4 ffu dose of Mixohipra-FSA; 10× M-FSA—vaccinated with 2.90×10^5 ffu dose of Mixohipra-FSA; 1× M-H—vaccinated with 1.95×10^4 ffu dose of Mixohipra-H; 10× M-H—vaccinated with 1.95×10^5 ffu dose of Mixohipra-, 1×-M-RHD—vaccinated with 1 dose of Myxo RHD-PLUS; Not vac. Not vaccinated; NT—non tested; ND—non detected H—Iberian hare R-wild rabbit.

4. Discussion

The objectives established for this study were substantially different for the two-animal species. For the Iberian hare, the main objective was to investigate if commercially available MYXV vaccines for use in rabbits might constitute a prophylactic tool for hares until a hare specific vaccine is available. The objectives for the wild rabbit were to prove the efficacy of commercial rabbit vaccines in wild rabbits against the recently emerged ha-MYXV (either isolated from Iberian hare or wild rabbit) and to investigate the susceptibility of wild rabbits to the ha-MYXV isolated from the Iberian hare.

Several vaccines against myxomatosis are currently available and can show protection when used properly in wild rabbits [21]. However, there are some disagreements in determining the effectiveness of vaccination campaigns in the wild as a management measure [22]. Vaccination campaigns of wild rabbits against myxoma virus are usually "blind", and non-systematic: vaccines are often administrated to the animals regardless of their sex, age or serological status [23].

The commercial myxomatosis vaccines contain live attenuated virus because multiplication of the virus, despite being limited, is important for the induction of a robust immune response, comprising also cellular immunity which is key for protection to myxomatosis [24]. As commercial vaccines against myxomatosis contain classic MYXV strains (e.g., VMI 30 strain) or a Shope fibroma virus strain, which are different from ha-MYXV to which the Iberian hare is highly susceptible, low efficacy of commercial rabbit vaccines is expected, given the absence of vaccine virus multiplication in hare cell cultures (personal communication).

The greatest limitation of this study was imposed by the small number of hares and wild rabbits included in the trial (three per group), intended to reduce to a minimum the number of animals, given the current critical situation of these populations and ethical issues. Despite this assumed constraint, our results showed that hares vaccinated with two commercially available vaccines (Mixohipra-FSA and Mixohipra-H) in the conditions recommended for rabbits, did not seroconvert robustly. Nor did the vaccinated hares gain protection against challenge with ha-MYXV, even when a very low dose of challenge virus (100 ffu) was used, close to that used in previous rabbit studies [25,26]. In fact, with this standard vaccination protocol, all vaccinated hares developed severe disease after challenge, similarly to the non-vaccinated controls.

Interestingly, 2 out of the 3 hares vaccinated with a 10-fold higher dose as recommended for domestic rabbits of Mixohipra-FSA, and a 10-fold higher dose of Mixohipra-H 21 days later, induced a satisfactory humoral response against MYXV. However, there was no remarkable change in antibody titres after the Mixohipra-H boost, which seems to indicate that vaccination with Mixohipra-FSA alone might be sufficient to induce protective immunity in hares. It is worth noting that the higher doses were not accompanied by any general or local (inoculation site) reactions. Furthermore, the haematological and biochemical data of the groups of hares vaccinated with the lower doses revealed no differences in globulins, contrasting with the decrease in the albumin/globulin ratio, in the case of hare #043 and hare #044 (H-G3), vaccinated with the 10-fold vaccine doses.

This difference in humoral response against these two vaccines, with a higher response against a heterologous vaccine, may be explained, among other reasons, by the fact that Mixohipra-FSA is an adjuvanted (attapulgite) vaccine and Mixohipra-H does not contain adjuvant, according to the manufacturer's documentation. However, further studies are needed to evaluate this hypothesis, namely by testing seroconversion after two successive administrations of Mixohipra-H, without a previously inoculation of Mixohipra-FSA.

The efficient containment of the Iberian hare for clinical evaluation and sampling is extremely difficult and represents a high risk of self-injury (vertebral fracture) due to sudden movements. Therefore, the subcutaneous route was chosen for all administrations considering that intradermal administration implies the total immobilization of the animal for a few minutes to allow the administration of an invariable dose in all animals.

Interestingly, hares #042 and #233 did not produce any disease after the first challenge trial (100 ffu), neither seroconverted after vaccination (#042) nor virus challenge (#042 and #233), showing a potential difference in the genetic susceptibility of some animals. No signs of cervical lymph node reaction were detected after the virus challenge. This can also mean that the subcutaneous route is not the ideal primary site of Myxoma virus multiplication or antigen presentation. Several studies have shown the lower effectiveness of the subcutaneous route in inducing immunity compared with the intradermal route [27,28]. The intradermal route allows a longer contact between the antigen and the antigen-presenting cells with a high number of dendritic cells in the derma compared with the subcutaneous tissue [29]. When Myxoma virus is inoculated intradermically, it can enter directly by lymphatic vessels for transport to antigen-presenting cells in the lymph nodes [30]. Considering the specificity of MYXV to epithelial cells, the delivery to the epidermis or dermis may result in superior and quick immune responses when compared to muscle and subcutaneous tissues [28]. This explanation can be also applied to the inoculation of virus during the challenge. Dalton et al. [27] found seropositivity of only 16.6–54% of rabbits after subcutaneous vaccination with a delay in seroconversion of these animals. However, in these two hares (#233 and #042), the second inoculation with 1000 ffu induced the typically fatal disease. This can mean that 100 ffu might be a low dose for a viral challenge by subcutaneous route, and is why a dose of 1000 ffu was used in the challenge carried out on rabbits, bearing in mind that this test was made after that of hares. Studies carried out in rabbits used different viral doses, namely $2 \times 10^{5.4}$ TCID50 (around $1.4 \times 10^{5.4}$ ffu) [31], unknown viral load [32] inoculated subcutaneously, or a dose of around 10^2 to 10^4 [33–39] inoculated intradermically.

In wild rabbits, both Mixohipra-H and Nobivac Myxo-RHD PLUS vaccines, administered in the dose recommended for domestic rabbits, induced a humoral response and completely protected the animals from experimental infection, as none of the rabbits' showed signs of disease after challenge. The wild rabbit is the same species as experimental, pet and industrial rabbits so it is expected that a similar response against the vaccination is developed, allowing the vaccine virus infection and replication in this species. According to the previous statement, the humoral and cellular response against the vaccine strain is expected to be protective against the naturally recombinant virus, taking into account that most of (all) the antigenic epitopes of classic MYXV are conserved in this recombinant virus.

Non-vaccinated rabbits succumbed to inoculation with ha-MYXV either isolated from Iberian hare (38455PT18) or wild rabbit (20545PT20), demonstrating, for the first time, the susceptibility of *Oryctolagus cuniculus* to the recombinant virus directly isolated from Iberian hare. Although these two strains (38455PT18 and 20545PT20) are still being fully characterized, the disruptive insertion of the M009L gene is conserved in both as well as the insertion affecting the M152R gene (Serp-3), a known virulence factor of MYXV [40].

Despite some differences were observed between the animals that developed severe myxomatosis (Tables 3–5), it was not possible to establish any clinical or lesional pattern between the hares of the different groups due to the small size of our sample, as it is likely that the differences found are due to natural inter-individual variability. However, the differences between the anatomopathological patterns found in hares and in rabbits was evident, probably as a result of the more rapid clinical evolution in the latter.

The macroscopic and histopathologic lesions found in hares and rabbits were similar to those described previously [1,3,7,8,19].

Myxomas (tumour-like lesions) were neither found in the skin of the hare or rabbit used in the three studies, nor in wild naturally infected with ha-MYXV [7], although they have already been found in domestic rabbits infected with ha-MYXV [8]. Furthermore, myxomas are not always present in wild hares found dead with ha-MYXV, being only present in around 30% of hares [6–8,20]. According to classic myxoma virus virulence grade classification [18,41,42], mortality of 100% in seronegative hares would correspond to Grade I viruses. However, in this assay, the average survival time was of 17.8 ± 8.5 days after symptoms onset and not ≤ 13 days as in rabbits infected in Grade I viruses, suggesting

that this classification is not appropriate for the Iberian hare. More studies are therefore needed to understand these differences.

All the immunized hares and rabbits showed a moderate to high increase of the antibody titres after the challenge, showing a non-sterilizing immunity. Interestingly, rabbit #10, which acquired immunity by recovering from a natural infection, showed no response after inoculation of 100 ffu of virus, with a small increase in antibodies after the second inoculation.

PCR based viral quantification revealed loads compatible with what was previously described [3,7,8]. As expected [7,8,20], a higher viral load was found in the skin (eyelid and lips) and external genitalia, of both hares and rabbits. However, a variable load of virus was found in various organs, proving systemic dissemination. Relatively high virus loads were also found in the central nervous system (brain and spinal cord), as reported previously for the classic virus strains [18].

The viral loads of ha-MYXV found in hares and rabbits were similar, regarding titre and distribution in different organs, suggesting similar pathophysiology and organic distribution of the virus in both species (Table 6), and independent from the dose of virus inoculated (100 or 1000 ffu). However, since the animals were not euthanized and did not die on the same day, the different periods of viral replication hamper further conclusions.

However, an important difference to highlight is the higher viral load (10–100× higher titres) in the lungs of rabbits compared to hares, which may be related to the fact that the clinical course in rabbits was more acute. This was true even when comparing the rabbits with the two hares (#042 and #233) infected with 1000 ffu.

This difference was particularly notable in animals inoculated with wild rabbit strain (#449 and #451, inoculated with 20545PT20), whose external lesions were scarcely evident, and that succumbed as a result of pulmonary alterations and accumulation of pleural effusion. These two animals died 12 days after virus inoculation and 4 days after the onset of symptoms. According to classic myxoma virus virulence grade classifications [18,41,42], these two virus strains can be classified as a Grade 1 (the highest virulence).

No particularly important bacteriological pathology was found in hares' histopathology, and opportunistic bacteria were generally found, probably as a result of immunosuppression caused by the myxoma virus [43]. The bacteriological findings were surprising, revealing the presence of several bacteria species in leporids for the first time, namely *Stenotrophomonas maltophilia*, *Enterococcus gallinarum* and *Vibrio vulnificus*, and confirming that secondary bacterial infections may have contributed significantly to the dead of the animals (Table 5). *Staphylococcus equorum* was also reported in nasal samples from wild rabbits in Azores, Portugal [44], but was never reported in hares, including the Iberian hare. *Stenotrophomonas maltophilia* was never reported in rabbits or hares, being an aerobic, non-fermentative, Gram-negative bacterium, uncommon and difficult to treat in humans [45]. *Enterococcus gallinarum* was also never reported in hares or rabbits, also being of zoonotic importance [46,47]. The *Vibrio vulnificus* is a multi-host opportunistic bacteria [48] never reported in leporids. This bacteria leads to human mortality rates of 50% by sepsis and of 17% due to wound infection [49]. Besides infection is rare, this species is responsible for the most deaths caused by Vibrios [49]. The hare species are known for their reservoir potential for many other emerging or re-emerging pathogens of public health importance, namely *Yersinia* spp., *Brucella* spp., and *Francisella tularensis* (reviewed in [50]).

The following conclusions can be taken from this study: (i) the Iberian hare is not protected from mortality due to myxomatosis by Mixohipra-FSA and/or Mixohipra-H according to the dose used in domestic rabbits, (ii) it is possible to protect the Iberian hare from mortality due to myxomatosis using a higher dose of Mixohipra-FSA, (iii) the commercial vaccines Mixohipra-H and Nobivac Myxo-RHD PLUS protect the wild rabbit effectively against natural recombinant myxoma virus strains and (iv) the wild rabbit is susceptible to ha-MYXV directly isolated either from the wild rabbit or the Iberian hare.

This finding indicates that wild rabbits may contribute to the spread of ha-MYXV in hares. As two commercial myxoma vaccines (Mixohipra-FSA and Mixohipra-H) showed no

efficacy in hares when using commercially recommended dosages, it is urgent to develop a robust vaccine for the Iberian hare or to investigate vaccine efficacy in hares of other commercial myxoma vaccines.

Institutional Review Board Statement: The animal study protocol was carried out in line with the measures identified in the National Plan for the control of rabbit haemorrhagic disease 2 (Dispatch 4757/2017, 31 May), operated through Project +Coelho 2, and approved by the National Authority for Animal Health (DGAV, Nr 79/ECVPT/20145) according to the National legislation (Decree-Law No. 113/2013, 7 August) after a positive declaration from the independent Advisory Body Responsible for Animal Welfare (ORBEA—INIAV, I.P.).

Informed Consent Statement: Not applicable.

Data Availability Statement: All data are presented in this study, there are no additional data.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Águeda-Pinto, A.; De Matos, A.L.; Abrantes, M.; Kraberger, S.; Risalde, M.A.; Gortázar, C.; McFadden, G.; Varsani, A.; Esteves, P.J. Genetic Characterization of a Recombinant Myxoma Virus in the Iberian Hare (*Lepus granatensis*). *Viruses* **2019**, *11*, 530. [CrossRef] [PubMed]
2. Dalton, K.P.; Martín, J.M.; Nicieza, I.; Podadera, A.; De Llano, D.; Casais, R.; Gimenez, S.; Badiola, I.; Agüero, M.; Duran, M.; et al. *Myxoma virusjumps* species to the Iberian hare. *Transbound. Emerg. Dis.* **2019**, *66*, 2218–2226. [CrossRef]
3. Carvalho, C.L.; Dos Santos, F.A.A.; Monteiro, M.; Carvalho, P.; Mendonça, P.; Duarte, M.D. First cases of myxomatosis in Iberian hares (*Lepus granatensis*) in Portugal. *Vet. Rec. Case Rep.* **2020**, *8*, e001044. [CrossRef]
4. Dos Santos, F.A.A.; Monteiro, M.; Pinto, A.; Carvalho, C.L.; Peleteiro, M.C.; Carvalho, P.; Mendonça, P.; Carvalho, T.; Duarte, M.D. First description of a herpesvirus infection in genus Lepus. *PLoS ONE* **2020**, *15*, e0231795. [CrossRef] [PubMed]

5. Duarte, M.D.; Carvalho, C.L.; Dos Santos, F.A.; Monteiro, J.; Monteiro, M.; Carvalho, P.M.; Mendonça, P.; Santos, P.T.; Melo, P.C. The Health and Future of the Six Hare Species in Europe: A Closer Look at the Iberian Hare. In *Lagomorpha Characteristics*; IntechOpen: London, UK, 2020.

6. García-Bocanegra, I.; Camacho-Sillero, L.; Caballero-Gómez, J.; Agüero, M.; Gómez-Guillamón, F.; Ruiz-Casas, J.M.; Díaz-Cao, J.M.; García, E.; Ruano, M.J.; de la Haza, R. Monitoring of emerging myxoma virus epidemics in Iberian hares (*Lepus granatensis*) in Spain, 2018–2020. *Transbound. Emerg. Dis.* **2020**, *68*, 1275–1282. [CrossRef] [PubMed]

7. Dos Santos, F.A.; Carvalho, C.; Pinto, A.; Rai, R.; Monteiro, M.; Carvalho, P.; Mendonça, P.; Peleteiro, M.; Parra, F.; Duarte, M. Detection of recombinant Hare Myxoma Virus in wild rabbits (*Oryctolagus cuniculus algirus*). *Viruses* **2020**, *12*, 1127. [CrossRef] [PubMed]

8. dos Santos, F.A.A.; Carvalho, C.L.; Monteiro, M.; Carvalho, P.; Mendonça, P.; Peleteiro, M.D.C.; Duarte, M.D. Recombinant myxoma virus infection associated with high mortality in rabbit farming (*Oryctolagus cuniculus*). *Transbound. Emerg. Dis.* **2020**, *68*, 2616–2621. [CrossRef]

9. Villafuerte, R.; Delibes-Mateos, M. Oryctolagus cuniculus. *IUCN Red List* **2019**, *8235.* [CrossRef]

10. Soriguer, R.; Carro, F.; Lepus granatensis. IUCN Red List 2019: E.T41306A2953195. Available online: https://www.iucnredlist.org/species/41306/2953195 (accessed on 10 January 2022).

11. Delibes-Mateos, M.; Ferreras, P.; Villafuerte, R. European rabbit population trends and associated factors: A review of the situation in the Iberian Peninsula. *Mammal Rev.* **2009**, *39*, 124–140. [CrossRef]

12. Reemers, S.; Peeters, L.; Van Schijndel, J.; Bruton, B.; Sutton, D.; Van Der Waart, L.; Van De Zande, S. Novel Trivalent Vectored Vaccine for Control of Myxomatosis and Disease Caused by Classical and a New Genotype of Rabbit Haemorrhagic Disease Virus. *Vaccines* **2020**, *8*, 441. [CrossRef]

13. Dos Santos, F.A.A.; Carvalho, C.L.; Peleteiro, M.C.; Gabriel, S.I.; Patrício, R.; Carvalho, J.; Cunha, M.V.; Duarte, M. Blood collection from the external jugular vein of *Oryctolagus cuniculus* algirus sedated with midazolam: Live sampling of a subspecies at risk. *Wildl. Biol.* **2019**, *2019*, 1–10. [CrossRef]

14. Smallwood, S.E.; Rahman, M.M.; Smith, D.W.; McFadden, G. Myxoma Virus: Propagation, Purification, Quantification, and Storage. *Curr. Protoc. Microbiol.* **2010**, *17*, 14A.1.1–14A.1.20. [CrossRef] [PubMed]

15. Baer, A.; Kehn-Hall, K. Viral Concentration Determination Through Plaque Assays: Using Traditional and Novel Overlay Systems. *J. Vis. Exp.* **2014**, e52065. [CrossRef] [PubMed]

16. dos Santos, F.A.A.; Carvalho, C.L.; Parra, F.; Dalton, K.P.; Peleteiro, M.C.; Duarte, M.D. A Quadruplex qPCR for Detection and Differentiation of Classic and Natural Recombinant Myxoma Virus Strains of Leporids. *Int. J. Mol. Sci.* **2021**, *22*, 12052. [CrossRef]

17. dos Santos, F.A.; Carvalho, C.; Peleteiro, M.; Parra, F.; Duarte, M. A Versatile qPCR for Diagnosis of Leporid Gammaherpesvirus 5 Using Evagreen® or Taqman® Technologies. *Viruses* **2021**, *13*, 715. [CrossRef] [PubMed]

18. Fenner, F.; Ratcliffe, F.N. *Myxomatosis*; Cambridge Universuty Press: London, UK, 1965.

19. García-Bocanegra, I.; Camacho-Sillero, L.; Risalde, M.A.; Dalton, K.P.; Caballero-Gómez, J.; Agüero, M.; Zorrilla, I.; Gómez-Guillamón, F. First outbreak of myxomatosis in Iberian hares (*Lepus granatensis*). *Transbound. Emerg. Dis.* **2019**, *66*, 2204–2208. [CrossRef] [PubMed]

20. Duarte, M.; Carvalho, C.L.; Santos, F.A.; Monteiro, J.; Gomes, J.; Alves, P.C.; Esteves, P.J.; Abrantes, J.; Lopes, A.M.; Monterroso, P.; et al. +Coelho2: Desenvolvimento e implementação de medidas práticas impulsionadoras da recuperação dos leporídeos silvestres em Portugal. In *Relatório de Atividades na Época Venatória 2018–2019 e 2019–2020*; Instituto Nacional de Investigação Agrária e Veterinária, I. P. (INIAV, I. P.): Oeiras, Portugal, 2020; Available online: https://www.iniav.pt/images/Noticias/Coelho2_Final-Report-web.pdf (accessed on 10 January 2022).

21. Guitton, J.-S.; Devillard, S.; Guénézan, M.; Fouchet, D.; Pontier, D.; Marchandeau, S. Vaccination of free-living juvenile wild rabbits (*Oryctolagus cuniculus*) against myxomatosis improved their survival. *Prev. Veter- Med.* **2008**, *84*, 1–10. [CrossRef]

22. Cabezas, S.; Calvete, C.; Moreno, S. Vaccination Success and Body Condition in the European Wild Rabbit: Applications for Conservation Strategies. *J. Wildl. Manag.* **2006**, *70*, 1125–1131. [CrossRef]

23. Ferreira, C.; Ramírez, E.; Castro, F.; Ferreras, P.; Alves, P.C.; Redpath, S.; Villafuerte, R. Field experimental vaccination campaigns against myxomatosis and their effectiveness in the wild. *Vaccine* **2009**, *27*, 6998–7002. [CrossRef]

24. Marlier, D. Vaccination strategies against myxomavirus infections: Are we really doing the best? *Tijdschr. Diergeneeskd.* **2010**, *135*, 194–198.

25. Kerr, P.J.; Merchant, J.C.; Silvers, L.; Hood, G.M.; Robinson, A.J. Monitoring the spread of myxoma virus in rabbit *Oryctolagus cuniculus* populations on the southern tablelands of New South Wales, Australia. II. Selection of a strain of virus for release. *Epidemiology Infect.* **2003**, *130*, 123–133. [CrossRef] [PubMed]

26. Ashton, L.V.; Quackenbush, S.L.; Castle, J.; Wilson, G.; McCoy, J.; Jordan, M.; MacNeill, A.L. Recombinant Myxoma Virus Expressing Walleye Dermal Sarcoma Virus or fC Is Attenuated in Rabbits. *Viruses* **2020**, *12*, 517. [CrossRef] [PubMed]

27. Dalton, K.; Nicieza, I.; de Llano, D.; Gullón, J.; Inza, M.; Petralanda, M.; Arroita, Z.; Parra, F. Vaccine breaks: Outbreaks of myxomatosis on Spanish commercial rabbit farms. *Veter- Microbiol.* **2015**, *178*, 208–216. [CrossRef] [PubMed]

28. Manev, I.; Genova, K.; Lavazza, A.; Capucci, L. Humoral immune response to different routes of myxomatosis vaccine application. *World Rabbit Sci.* **2018**, *26*, 149–154. [CrossRef]

29. Levin, C.; Perrin, H.; Combadiere, B. Tailored immunity by skin antigen-presenting cells. *Hum. Vaccines Immunother.* **2014**, *11*, 27–36. [CrossRef]

30. Kim, Y.C.; Jarrahian, C.; Zehrung, D.; Mitragotri, S.; Prausnitz, M.R. Delivery Systems for Intradermal Vaccination. *Intradermal Immun.* **2011**, *351*, 77–112. [CrossRef]

31. Psikal, I.; Smid, B.; Rodak, L.; Valicek, L.; Bendova, J. Atypical myxomatosis-virus isolation, experimental infection of rabbits and restriction endonuclease analysis of the isolate. *J. Veter- Med. Ser. B* **2003**, *50*, 259–264. [CrossRef]

32. Marshall, I.D.; Fenner, F. Studies in the epidemiology of infectious myxomatosis of rabbits: VII. The virulence of strains of myxoma virus recovered from Australian wild rabbits between 1951 and 1959. *J. Hyg.* **1960**, *58*, 485–488. [CrossRef]

33. Kerr, P.; Perkins, H.; Inglis, B.; Stagg, R.; McLaughlin, E.; Collins, S.; Van Leeuwen, B. Expression of rabbit IL-4 by recombinant myxoma viruses enhances virulence and overcomes genetic resistance to myxomatosis. *Virology* **2004**, *324*, 117–128. [CrossRef]

34. Kappler-Gratias, S.; Bucher, L.; Top, S.; Quentin-Froignant, C.; Desbois, N.; Bertagnoli, S.; Louison, M.; Monge, E.; Bousquet-Melou, A.; Lacroix, M.; et al. Antipoxvirus Activity Evaluation of Optimized Corroles Based on Development of Autofluorescent ANCHOR Myxoma Virus. *ACS Infect. Dis.* **2021**, *7*, 2370–2382. [CrossRef]

35. Boutard, B.; Vankerckhove, S.; Markine-Goriaynoff, N.; Sarlet, M.; Desmecht, D.; McFadden, G.; Vanderplasschen, A.; Gillet, L. The α2,3-Sialyltransferase Encoded by Myxoma Virus Is a Virulence Factor that Contributes to Immunosuppression. *PLoS ONE* **2015**, *10*, e0118806. [CrossRef] [PubMed]

36. Bárcena, J.; Morales, N.; Boga, A.; Parra, F.; Sa, M.; Lucientes, J.; Page, A.; Blasco, R.; Torres, J.M. Horizontal Transmissible Protection against Myxomatosis and Rabbit Hemorrhagic Disease by Using a Recombinant Myxoma Virus. *J. Virol.* **2000**, *74*, 1114–1123. [CrossRef] [PubMed]

37. OIE. Terrestrial Manual Myxomatosis. In *Lagomorpha*; OIE: Paris, France, 2018; pp. 1371–1388.

38. Kerr, P.J.; Cattadori, I.M.; Rogers, M.B.; Fitch, A.; Geber, A.; Liu, J.; Sim, D.G.; Boag, B.; Eden, J.-S.; Ghedin, E.; et al. Genomic and phenotypic characterization of myxoma virus from Great Britain reveals multiple evolutionary pathways distinct from those in Australia. *PLoS Pathog.* **2017**, *13*, e1006252. [CrossRef] [PubMed]

39. Spibey, N.; McCabe, V.J.; Greenwood, N.M.; Jack, S.C.; Sutton, D.; van der Waart, L. Novel bivalent vectored vaccine for control of myxomatosis and rabbit haemorrhagic disease. *Veter- Rec.* **2012**, *170*, 309. [CrossRef]

40. Guerin, J.-L.; Gelfi, J.; Camus, C.; Delverdier, M.; Whisstock, J.; Amardeihl, M.-F.; Py, R.; Bertagnoli, S.; Messud-Petit, F. Characterization and functional analysis of Serp3: A novel myxoma virus-encoded serpin involved in virulence. *J. Gen. Virol.* **2001**, *82*, 1407–1417. [CrossRef]

41. Fenner, B.Y.F.; Marshall, I.D. A comparison of the virulence for european rabbits (*Oryctolagus cuniculus*) of strains of Myxoma virus recovered in the field in Austral, Europe and America. *J. Hyg.* **1957**, *55*, 149–191. [CrossRef]

42. Fenner, F.; Woodroofe, G.M. Changes in the virulence and antigenic structure of strains of myxoma virus recovered from Australian wild rabbits between 1950 and 1964. *Aust. J. Exp. Biol. Med. Sci.* **1965**, *43*, 359–370. [CrossRef]

43. Jeklova, E.; Leva, L.; Matiasovic, J.; Kovarcik, K.; Kudlackova, H.; Nevorankova, Z.; Psikal, I.; Faldyna, M. Characterisation of immunosuppression in rabbits after infection with myxoma virus. *Veter- Microbiol.* **2008**, *129*, 117–130. [CrossRef]

44. Sousa, M.; Silva, V.; Silva, A.; Silva, N.; Ribeiro, J.; Tejedor-Junco, M.T.; Capita, R.; Chenouf, N.S.; Alonso-Calleja, C.; Rodrigues, T.M.; et al. Staphylococci among Wild European Rabbits from the Azores: A Potential Zoonotic Issue? *J. Food Prot.* **2020**, *83*, 1110–1114. [CrossRef]

45. Brooke, J.S. *Stenotrophomonas maltophilia*: An emerging global opportunistic pathogen. *Clin. Microbiol. Rev.* **2012**, *25*, 2–41. [CrossRef]

46. Barber, G.R.; Lauretta, J.; Saez, R. A Febrile Neutropenic Patient with *Enterococcus gallinarum* Sepsis Treated with Daptomycin and Gentamicin. *Pharmacother. J. Hum. Pharmacol. Drug Ther.* **2007**, *27*, 927–932. [CrossRef] [PubMed]

47. Li, X.; Fan, S.; Lin, X.; Liu, L.; Zheng, J.; Zhou, X.; Heep, A. The first case report of *Enterococcus gallinarum* meningitis in neonate. *Medicine* **2018**, *97*, e9875. [CrossRef] [PubMed]

48. Roig, F.J.; González-Candelas, F.; Sanjuan, E.; Fouz, B.; Feil, E.J.; Llorens, C.; Baker-Austin, C.; Oliver, J.D.; Danin-Poleg, Y.; Gibas, C.J.; et al. Phylogeny of *Vibrio vulnificus* from the Analysis of the Core-Genome: Implications for Intra-Species Taxonomy. *Front. Microbiol.* **2018**, *8*, 2613. [CrossRef] [PubMed]

49. Horseman, M.A.; Surani, S. A comprehensive review of *Vibrio vulnificus*: An important cause of severe sepsis and skin and soft-tissue infection. *Int. J. Infect. Dis.* **2011**, *15*, e157–e166. [CrossRef] [PubMed]

50. Tsokana, C.N.; Sokos, C.; Giannakopoulos, A.; Birtsas, P.; Valiakos, G.; Spyrou, V.; Athanasiou, L.V.; Burriel, A.R.; Billinis, C. European Brown hare (*Lepus europaeus*) as a source of emerging and re-emerging pathogens of Public Health importance: A review. *Veter- Med. Sci.* **2020**, *6*, 550–564. [CrossRef] [PubMed]

Did MYXV in hares lead to the reactivation of other pathogens?

Scientific publication 14

First description of a herpesvirus infection in genus Lepus

■■

Immunosupression is pivotal in the emergence of concomitant subclinical infections leading to the reactivation of latent viruses such as herpesviruses. During the study of myxomatosis emergence in Iberian hare, pathological lesions distinct from those observed in myxomatosis, and compatible with a herpesvirus infection, were identified. Following the observation of herpetic vesicles, LeHV-5 was described, constituing the first herpesvirus identified in the genus Lepus.

PLOS ONE

First description of a herpesvirus infection in genus Lepus

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Abstract

During the necropsies of Iberian hares obtained in 2018/2019, along with signs of the nodular form of myxomatosis, other unexpected external lesions were also observed. Histopathology revealed nuclear inclusion bodies in stromal cells suggesting the additional presence of a nuclear replicating virus. Transmission electron microscopy further demonstrated the presence of herpesvirus particles in the tissues of affected hares. We confirmed the presence of herpesvirus in 13 MYXV-positive hares by PCR and sequencing analysis. Herpesvirus-DNA was also detected in seven healthy hares, suggesting its asymptomatic circulation. Phylogenetic analysis based on concatenated partial sequences of DNA polymerase gene and glycoprotein B gene enabled greater resolution than analysing the sequences individually. The hare' virus was classified close to herpesviruses from rodents within the Rhadinovirus genus of the gammaherpesvirus subfamily. We propose to name this new virus Leporid gammaherpesvirus 5 (LeHV-5), according to the International Committee on Taxonomy of Viruses standards. The impact of herpesvirus infection on the reproduction and mortality of the Iberian hare is yet unknown but may aggravate the decline of wild populations caused by the recently emerged natural recombinant myxoma virus.

1. Introduction

The Iberian hare (*Lepus granatensis*), also known as Granada hare, is an endemic specie of the Iberian Peninsula whose populations are considered stable by the IUCN holding a '*minor concern*' conservation status [1].

Lepus granatensis is the only hare species found in Portugal and the most widespread in the Iberia [2], therefore, highly relevant for biodiversity preservation and hunting activity in both countries, particularly for greyhound racing.

Contrarily to the wild rabbit, which drastic decline has been linked, among other factors, to viral epizooties, until recently, the Iberian hare was not affected by viral diseases. Environmental and anthropogenic factors, however, have had a negative impact on both hare and wild-rabbit populations.

Lepus granatensis was considered naturally resistant to myxomatosis, which is endemic in Iberian Peninsula since 1956 [3], despite very sporadic reports of the disease in the European brown hare (*L. europaeus*), namely in France and Ireland [4–6]. However, during the summer and autumn of 2018 outbreaks of myxomatosis in the Iberian hare were reported in Spain [7] and Portugal [8], respectively.

No other diseases of viral origin have been described in the Iberian hare, including those caused by herpesviruses, which may have a fatal outcome in rabbits [9].

Until now, four herpesviruses have been identified in leporids: Leporid herpesvirus 1 (LeHV-1), Leporid herpesvirus 2 (LeHV-2), Leporid herpesvirus 3 (LeHV-3) and Leporid herpesvirus 4 (LeHV-4) (Table 1). Of these, the most common naturally occurring herpesvirus

Table 1. Summary of the characteristics of the four herpesviruses identified in leporids.

Type	Subfamily		Common Name	Host	Physiopathology	Isolation
LeHV-1	γ	Not attributed [a]	Cottontail herpesvirus	*Sylvilagus floridanus*		Isolated from primary kidney cells cultures of *Sylvilagus floridanus* [13].
						No report of disease in domestic rabbits, since *Oryctolagus cuniculus* is not infected [10].
LeHV-2	γ	Not attributed [a]	Herpesvirus cuniculi	*Oryctolagus cuniculus*	Some evidences of a subclinical encephalitis in infected New Zealand white rabbits [30].	Isolated in 1968 from kidneys of apparently healthy *Sylvilagus floridanus* [31].
						Oryctolagus cuniculus is the natural host where infection is asymptomatic [32]
LeHV-3	γ	Not attributed [a]	Herpesvirus sylvilagus	*Sylvilagus floridanus*	Lymphoproliferative disease and tumour-like lesions in the lymph nodes, kidney, spleen, and liver [31,33].	Isolated from primary kidney cells cultures of Sylvilagus floridanus [13].
						Oryctolagus cuniculus is not infected
						Not isolated in WI-38, HeLa, Chang's conjunctiva, human amnion (FL), green monkey kidney (Vero), primary rhesus monkey kidney, primary hamster kidney, BHK-21, primary mouse embryo, and primary chick embryo [33]. Isolated in DRK-3 cells [33].
						CPE appear after 10–15 days of inoculation. Infected cells show focal areas of round and distorted cells, and in 1–2 days, emerged syncytial masses containing 50 or more nuclei [31].
						H&E coloration show typical type A intranuclear inclusions in the infected cells. Complete cell destruction occurred after a 5 to 7-days period [31].
LeHV-4	α	Attributed [b]		*Oryctolagus cuniculus*	Lethargy, anorexia, conjunctivitis, fever, and abortion. Haemorrhagic dermatitis, splenic necrosis, hepatic necrosis, and multifocal pulmonary haemorrhage and oedema. Distinctive glassy eosinophilic herpetic intranuclear inclusion bodies were observed in the skin fibroblasts? And mesenchymal cells of the spleen and lung [9,34].	Isolated in rabbit skin (RS), RK13 and Vero cells [9]. CPE characterized by syncytium formation, cell enlargement, and cell lysis, similar to human herpesvirus type 1 (HHV-1). Jin et al. [9] verified that in rabbits inoculated with LHV-4, the appendix, sciatic nerve, kidney, adrenal gland, and many other organs were positive for the virus at the 5-days post infection(dpi), while at the 14 dpi only trigeminal ganglia eye and tonsil were positive.
LeHV-5	γ	Not attributed	Iberian hare herpesvirus	*Lepus granatensis*	Described in the results of this work	

[a]Not attributed by the ICTV; Viruses which may be members of the genus Rhadinovirus [35] but have not been approved as species

[b]Leporid alphaherpesvirus //Leporid Herpesvirus 4

infections identified in rabbits are LeHV-2 and LeHV-3 (reviewed by [9]), which alongside LeHV-1 belong to the Gammaherpesvirinae subfamily. Conversely, LeHV-4 is a member of the Alphaherpesvirinae subfamily. These distinct herpesviruses have a broadly variable impact on the European rabbit, with LeHV-2 and LeHV-3 infections usually passing unnoticed [10], while LeHV-4 is far more aggressive, causing fatal infections [9].

Herpesviruses are enveloped viruses, of 200–250 nm in diameter, organised in four concentric layers [11], 1) a core with the linear dsDNA genome, 2) T = 16 icosahedral capsid with about 125nm of diameter surrounded by a 3) proteinaceous tegument that contains many virus-coded proteins and enclosed in a 4) lipid envelope containing several viral glycoproteins. Morphologically, herpesviruses are distinct from all other viruses [12], and therefore easily recognised by electron microscopy.

Herpesviruses belong to order Herpesvirales that comprises three families, namely the Herpesviridae family, which includes more than 100 viruses of mammals, birds and reptiles and whose members have large genomes ranging from 125 to 290kb [13], the Alloherpesviridae family, which includes the fish and frog viruses, and the Malacoherpesviridae family, which contains the bivalve virus [12].

The Herpesviridae family includes the subfamilies Alphaherpesvirinae, Betaherpesvirinae and Gammaherpesvirinae. Their members have different biologic properties and distinct classification, supported by phylogeny. The Gammaherpesvirinae subfamily is divided into four genera, namely *Macavirus*, *Percavirus*, *Lymphocryptovirus*, and *Rhadinovirus* [12].

While the subfamily Alphaherpesvirinae causes rapid lysis in cell culture, members of Betaherpesvirinae grow slowly inducing the formation of giant cells in culture, and Gammaherpesvirinae typically infect lymphoid tissue, meaning a primary tropism for lymphoid lineage cells [14], which can lead to lymphoproliferative diseases [9] and oncogenesis [13].

In this study, we investigated the presence of herpesvirus in myxoma virus (MYXV)-positive hares that alongside, the typical myxoma virus-induced skin lesions, presented other lesions in the genitalia, eyelids, lips and nose suggestive of herpesvirus infection.

To unveil the prevalence of herpesvirus in the hare populations, healthy hares were also investigated.

2. Materials and methods

1. Sample

A total of 38 Iberian hares, none sacrificed for the purpose of this study, were investigated within the scope of a national surveillance program (Dispatch 4757/17, 31th may) [8] in action since August 2017. Of these, 16 were males, 20 were females, and two failed sex-determination. Eighteen hares were legally hunted by the Hunting Associations during the 2018/2019 season (October to December), authorized by permits from the National Forest Authority, the Institute for Nature Conservation and Forests (ICNF), while 20 were found dead in the field between October 2018 and June 2019. None of the authors were responsible for the death of the animals. Hunted and hares found dead were sampled in six Districts of mainland Portugal, namely Setúbal, Santarém, Beja, Évora, Portalegre and Faro, (Fig 1).

2. Necropsy and histopathology

Cadavers were necropsied and spleen, liver, lung, duodenum and skin samples (namely scrotum, lips and nose) were collected for virology, bacteriology and histopathology. The entire gastrointestinal tract was taken for parasitological analysis. From hunted hares, only spleen, liver and lung samples were received at the laboratory.
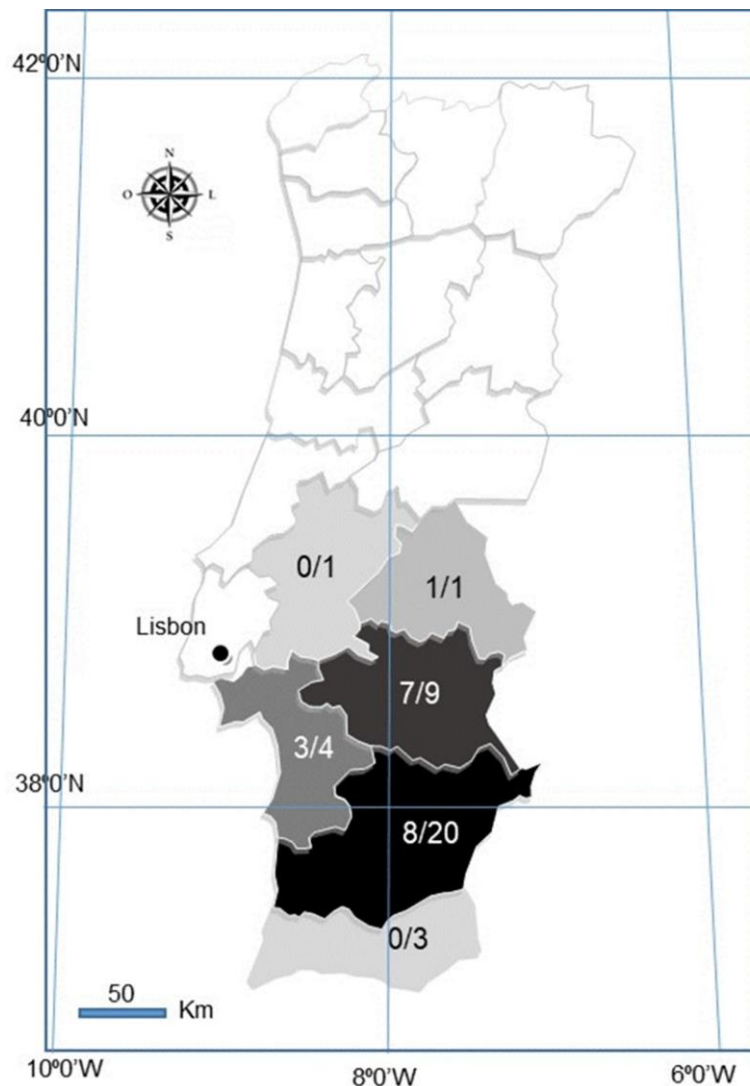
Fig 1. Map of Portugal showing the geographic origin of the 38 LeHV-5 positive hares over the total sampling per district. White coloured Districts were not sampled. Darker shades correspond to higher positivity.

For histopathology, skin and genitalia fragments were fixated in 10% neutral buffered formalin, routinely paraffin embedded, sectioned at 4 μm, and stained with Hematoxylin and Eosin (H&E).

2.3. Transmission electron microscopy

The fragments selected for transmission electron microscopy (TEM) were formalin fixated for 48h or on a solution 0.1M sodium cacodylate (Sigma©) containing 2.5% gluteraldehyde (Sigma©) for 72h. When the samples were already embebed in paraffin, the regions of interest were extracted from the block, sliced smaller than ~1mm^3 with a scalpel blade into two separate viles, and washed thoroughly in xylene. After rehydration using decreasing concentrations of ethanol, fragments were washed in 0.1M cacodylate buffer [15]. Samples were then post-fixed with 2% osmium tetroxide (EMS) for 30min, and stained in block with 1% Millipore-filtered uranyl acetate (Agar Scientifics), after which they were dehydrated in increasing

concentrations of ethanol, infiltrated and embedded in EMBed-812 hard (EMS). Polymerization was performed at 60˚C for 2 days. Ultrathin sections were cut either in a UC7 ultramicrotome or in a Reichert ultracut E ultramicrotome (Leica), collected to 1% formvar coated copper slot grids (Agar scientific), stained with uranyl acetate and lead citrate (Sigma) and examined in a H-7000 transmission electron microscope (Hitachi) at an accelerating voltage of 100 kV or Jeol 1400plus transmission electron microscope at an accelerating voltage of 120 kV. Digital images were obtained using a Megaview mid mount digital camera (Olympus) or using a AMT XR16 bottom mid mount digital camera (AMT©). The sections were systematically analysed using AMT© software and several high and low magnifications were acquired.

4. Bacteriological and parasitological examination

Liver, spleen and lung samples were analysed using standard bacteriological methods. Parasitological examination of the intestine was carried out resourcing to direct wet mount, sedimentation and filtration techniques.

To investigate the presence of Enterobacteriacea the ID 32E (Biomerieux1) was used, while for non-Enterobacteriaceaethe API 20NE kit (Biomerieux1) was utilised. To test the presence of Streptococcus and Staphylococcus, the ID 32 STREPT (Biomerieux1) and the ID 32 STAPH kits (Biomerieux1) were used, respectively. The API CORYNE (Biomerieux1) kit was utilized for the identification of Corynebacteria and coryne-like organisms. To investigate the presence of Salmonella, peptone water and Rappaport Vassiliadis semi solid culture media were used. The agarose SMID2 and XLD culture media were utilized, whenever there was a suspicion of Salmonella. Other culture media used for bacterial identification in the samples included the MacConkey agar and the Blood agar culture media.

5. Virological examination

For nucleic acid extraction, fresh samples of liver and spleen were homogenised at 20% with phosphate buffered saline (PBS) and clarified at 3000g for 5 min. Total DNA and RNA were extracted from 200μl of the clarified supernatants, using the MagAttract 96 cador Pathogen Kit in a BioSprint 96 nucleic acid extractor (Qiagen, Hilden, Germany), according to the manufacturer's protocol.

All the animals were tested for rabbit haemorrhagic disease virus 2 (RHDV2) and MYXV by real time PCR systems described by Duarte *et al* (2015) [12] and Duarte *et al* (2014) [22], respectively. The presence of LEHV-4 was investigated by using the PCR described by Jin *et al* (2008) [9]. A generalist nested PCR directed to the herpesviral *DNA polymerase* that allows the detection of herpesviruses of different subfamilies by Van Devanter *et al*. (1996) [16] was also used.

The glycoprotein B gene was partially amplified using the GH1 system described previously [17].

Amplifications were carried out in a Bio-Rad CFX96™ Thermal Cycler (Bio-Rad Laboratories Srl, Redmond, USA), using the One Step RT-PCR kit (Qiagen, Hilden, Germany) for RHDV2, and the HighFidelity PCR Master Mix (Roche Diagnostics GmbH, Mannheim, Germany), for MYXV and herpesvirus detection, respectively.

Information regarding these methods is summarized in Table 2.

6. Sequencing analysis

The PCR products were visualised in 2% horizontal electrophoresis agarose gel, purified using 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, Foster City,

Table 2. Molecular methods used for the detection of RHDV2 (Duarte et al, 2015), MYXV (Duarte et al, 2014), LeHV 4 [9] and a wide variety of herpesviral genomes from human and animal herpesviruses (Van Devanter, 1996).

Primer	Sequence (5'-3')	Target gene	Amplicon size (bp)	Amplification conditions	Reference
RHDV2-F	TGGAACTTGGCTTGAGTGTTGA	Vp60	127	50˚C -45min 95˚C-15min 50x (95˚C-15s 60˚C-30s 72˚C-30s)	[36]
RHDV2-R	ACAAGCGTGCTTGTGGACGG				
RHDV2-Probe	[FAM]-TGTCAGAACTTGTTGACATCCGCCC-[TAMRA]				
M000.5R/L-F	CGACGTAGATTTATCGTATACC	M000.5	125	95˚C- 10min 45X (95˚C-30s 50ºC-30s 60˚C-30s)	[37]
M000.5R/L-R	GTCTGTCTATGTATTCTATCTCC4				
MYXV-probe	[FAM]-TCTATGTCTGCCCGAGGATAGA-[TAMRA]				
LeHV-4 F1F	ATGACGCCCACCAACGTCTC GCACAGTGTGTGTTAGACG		1617 1162 945	95ºC-10min 35x (95º-15s 54ºC-20s 72ºC-3min) 72ºC-10min	[34]
LeHV-4 F2F	TGTGGCCAAGAACAACGATA				
LeHV-4 F3F					
LeHV-4 F1R	CATAGACCGTAGGCGGTTC				
LeHV-4 F2R	ACGTGAACAGGAACCGGTAG				
LeHV–4 F3R	CTAGAGGTCGTTCACCACCG				
DFA (F 1st round)	GAYT TYGCNAGYYTNTAYCC	DNA polymerase	215 to 315	94ºC- 10min 35x (94ºC-60s 46ºC-60s 72ºC-3min)	[16]
ILT (F1st round)	TCCTGGACAAGCAGCARNYS GCNMTNAA				
TGV (F1st round)	TGTAACTCGGTGTAYGGNTTYACNGGNGT				
KG1 (R 2nd round)	GTCTTGCTCACC AGNTCNACNCCYTT				
IYG (R 2nd round)	CACAGAGTCCGTRTCNCCRTADAT				
2759s	CCTCCCAGGTTCARTWYGCMT AYGA	gB	700 bp	94ºC- 10min 35x (94ºC-60s 46ºC-60s 72ºC-3min)	[17]
2762as	CCGTTGAGGTTCTGAGTGTAR TARTTRTAYTC				
2760s	AAGATCAACCCCAC(N/I)AG (N/I)GT(N/I)ATG		500bp		
2761as	GTGTAGTAGTTGTACTCCCTR AACAT(N/I)GTYTC				

CA, U.S.A). Nucleotide sequences were analysed and assembled into consensus sequences using the BioEdit version 7.2.5 software, and submitted to GenBank. Nucleotide sequences were translated using Mega X 10.1 software.

2.7. Phylogenetic analysis

Partial nucleotide (171bp) sequences of the viral *DNA polymerase* gene were aligned using the Clustal W with gap opening penalty and a gap extend penalty of 30 and 15, respectively. A phylogenetic analysis was conducted in MEGA X [18], using the model selected by Model function (MEGA X).

The evolutionary history of 28 partial DNA polymerase protein sequences of gammaherpesviruses was inferred by Maximum Likelihood. The Hasegawa-Kishino-Yano (HKY) model [19], which showed the lowest Bayesian Information Criterion (BIC) and Akaike Information Criterion corrected (AICc) values was used. A discrete Gamma distribution (G) was used to model evolutionary rate differences among sites. The rate variation model allowed for some sites to be evolutionarily invariable (I).

For more accurate phylogenetic analysis of the hare herpesvirus described is this manuscript, the partial nucleotide (171bp) sequences of the viral DNA polymerase gene catalytic subunit was concatenated with the partial (453bp) nucleotide sequences of the viral Glycoprotein B gene belonging to the same strain. The sequences were translated and aligned using the Clustal W with gap opening penalty and a gap extend penalty of 30 and 15, respectively. The

final alignment was edited to include all the sequences, corresponding to 570 nucleotides and 190 amino acids of length.

The Le Gascuel model (2008) [20], Gamma distributed with invariant sites (LG+G+I) was selected for the protein-based trees, according to BIC (14177.4) and AICC (13542.9) criteria. The analysis involved 45 amino acid sequences.

2.8. Herpesvirus isolation

Isolation of herpesvirus was attempted from organs of hares coinfected with MYXV and LeHV-5, namely from liver and spleen, penile and vulva samples. In addition, liver and spleen samples from two hares with single herpesvirus infection, were also used.

Samples were homogenized at 20% in phosphate-buffered saline containing penicillin, streptomycin and amphotericin B (antibiotic-antimycotic), used according to the manufacturer (Gibco, Life Technologies Corporation). Following centrifugation, the supernatant was filtered through a 0.45-μm-pore-size filter (Millipore Express) and used to inoculate sub 70% confluent Candrel R Feline Kidney (CRFK) epithelial cells (ATCC-CCL-94), Vero cells (ATCC No. CRL-1986), Rabbit Kidney (RK13) cells (ATCC-CCL-37) and Hella cells (ATCCNo. CRM-CCL-2)- RK13 cells grown in Eagle medium and the others in Dulbecco's modified Eagle's Media was supplemented with 10% foetal calf serum (Gibco), penicillin, streptomycin and amphotericin B (antibiotic-antimycotic used at 1:100), 50μg/ml gentamicin (Gibco). Cells were maintained at 37˚C under humidified atmosphere with 5% CO_2 and observed daily for cytopathic effect (CPE) by phase contrast microscopy. Three passages were carried out. The supernatant and cell pellet of each passage were tested for the presence of herpesvirus by PCR [17].

3. Results

1.Necropsy showed lesions compatible with herpesvirus infection in Iberian hares with myxomatosis

Overall, MYXV-positive hares revealed good body condition and, alongside typical myxomatosis lesions, necrosis of the genitalia was noticed in more than 70% of the hares studied. This lesion was more evident in males, affecting the penile glans, but was also observed in females inthe vulva. Other lesions observed in these hares included the presence of herpetic-like skin vesicles, uncommon in rabbits with myxomatosis.

Further investigation of the macroscopic lesions and histopathological patterns was carried out in hares co-infected with LeHV-5 and MYXV. At this time, we disclose the macroscopic and histopathological findings from two male hares found dead in November 2018 (#38455/18, hereafter designated hare-1) and August 2019 (#25456/19, hereafter designated hare-2).

Hare-1 presented with eyelids thickened by the accumulation of mucopurulent exudate and marked enlargement of the penis measuring 1.3x1cm in diameter (normal diameter is less than 0.5cm) and irregular surface (Fig 2 and 2A) lined with light-yellow dry exudate.

At the necropsy, hare-2 showed ulcerated multinodular thickening of the eyelids and lips. Accumulation of mucopurulent exudate in both eyes was also registered and a small vesicle was present in the lower lip (Fig 3).

2.   Histopathology

The dermis of hare-1 showed fusiform or stellate mesenchymal cell proliferation, surrounded by abundant extracellular matrix, scattered infiltration by lymphocytes and macrophages, and small aggregates of heterophiles, consistent with myxomatosis.

The penile epithelium of this hare was mostly necrotic and replaced by a thick band of necrotic cells, heterophils and red blood cells (Fig 2).

Severe heterophile infiltrations of the stroma, in either a diffuse pattern or multifocal aggregates, were also seen. In the stroma, there was also proliferation of pleomorphic spindle cells, with some nuclei almost filled with slightly eosinophilic inclusion bodies (Cowdry type A inclusions) (Fig 4), suggesting a nuclear replicating virus. These lesions, unexpected in myxomatosis, are compatible with herpesvirus.

In the skin of hare-2, a ballooning degeneration of keratinocytes was registered. Coalescent intra-epidermal and subepidermal vesicopustules (Fig 5) filled with fibrin and necrotic cells debris and multifocal detachment of the eyelids, lips and foreskin epidermis were seen. In the underlying dermis, multifocal haemorrhages, intense infiltration by heterophils and necrotic cells with accumulation of chromatin debris were present (Fig 5).

Below the dermis, accumulation of myxoid tissue with pleomorphic spindle cells, some of which showing rounded or oval and slightly eosinophilic intranuclear inclusion bodies, was observed (Fig 6). An infiltrate of mononucleated inflammatory cells and heterophils was present in skeletal muscle tissue.

3.3. Electron microscopy

Samples from hare-1 and hare-2 were further processed and analysed for TEM allowing the confirmation of the presence of herpesvirus in different tissues.
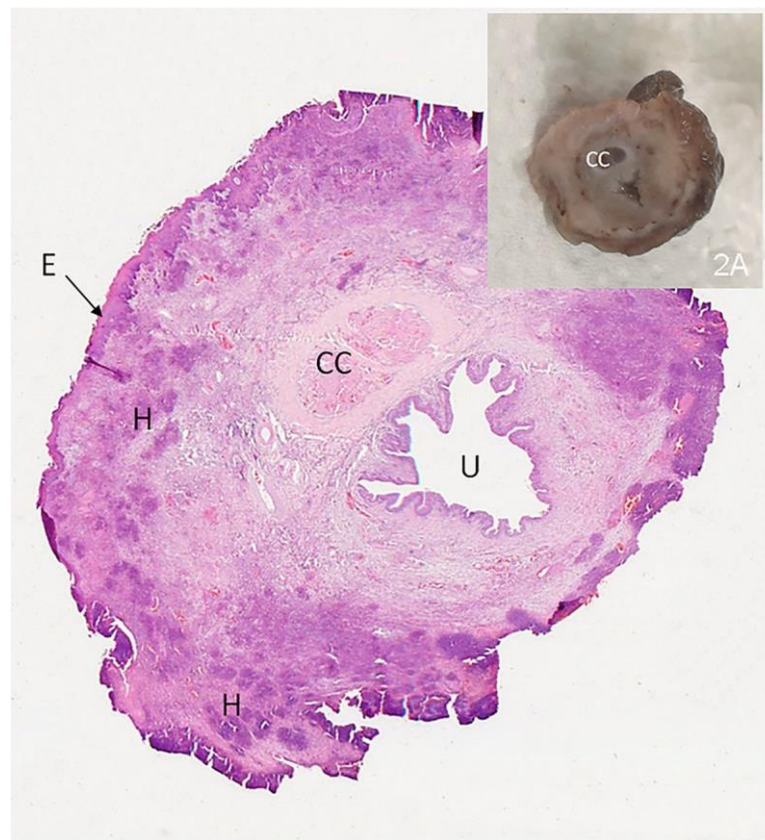
Fig 2. Penis of hare-1. 2- H&E staining showing several necrotic areas in the epithelium (E) and multifocal heterophils aggregates in the stromal tissue (H). *Corpus cavernosum* (CC); penile uretra (U). 4x. 2A - Cross section of penis after fixation- exuberant thickening of the penis.

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Fig 3. Hare-2 –Oedema of lips and nose with ulcerated nodules in the upper lip. A vesicle can be seen in the lower lip (arrow).

In the penile soft tissue of hare-1, spherical virions, with structure and dimensions compatible with herpesviruses, comprising an inner core packed into an icosahedral capsid, were observed in the nucleus of stromal cells (Fig 7), indicating nuclear replication (Fig 7B), which is an attribute of herpesviruses. The viral capsid contained a relatively small, asymmetrical, electron-dense region that probably represents the condensed DNA core. In this animal, also positive to myxomatosis, no MYXV particles were found in the samples processed.

3.4. Virological, bacteriological and parasitological results

None of the 38 hares investigated in this study tested positive to RHDV, RHDV2 or LEHV-4. Fifty percent of the hares were positive to LeHV-5, of which 68.4% (13/19) were also positive to MYXV.

Herpesvirus-DNA was also detected by PCR in the liver, spleen and lung samples of 41.2% (7/17) of the apparently healthy hunted hares that tested negative for MYXV. From this group of hares, no genitalia/skin samples were available for histopathology.
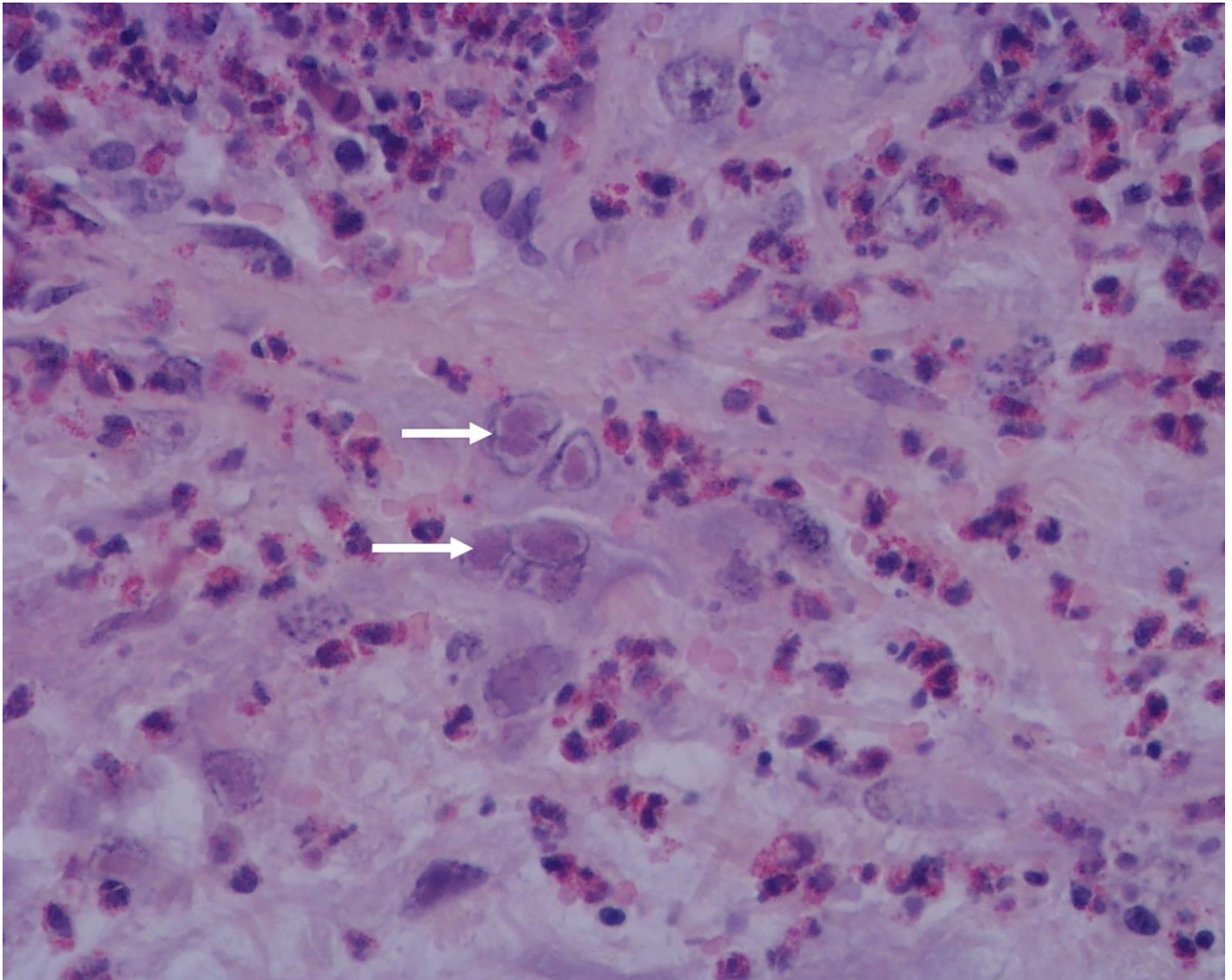
Fig 4. Penis of hare-1. Intranuclear inclusion bodies in mesenchymal cells (arrows) and moderate to severe infiltration by heterophils, H&E, 400x.

Six hares showed doubtful results from which four were MYXV-negative. Parasitological and bacteriological examinations did not reveal any infections that could justify the death of these animals.

3.5. Molecular characterisation of Iberian hare herpesvirus

For 50% of the animals (19/38), an amplicon ~225 bp-long, compatible with herpesvirus, was obtained in the pan-herpesvirus PCR [17]. For six hare samples only a weak band was generated, therefore were not considered for further analysis. The presence of herpesvirus was confirmed by sequencing analysis in 16 hares. For 11 of these amplicons, the nucleotide sequences obtained were independently edited to remove the primer targeting sequences and assembled. The consensus sequences (171 bp) showed 100% similarity to each other. Five of the obtained sequences were submitted to the GenBank (MN557129-33).
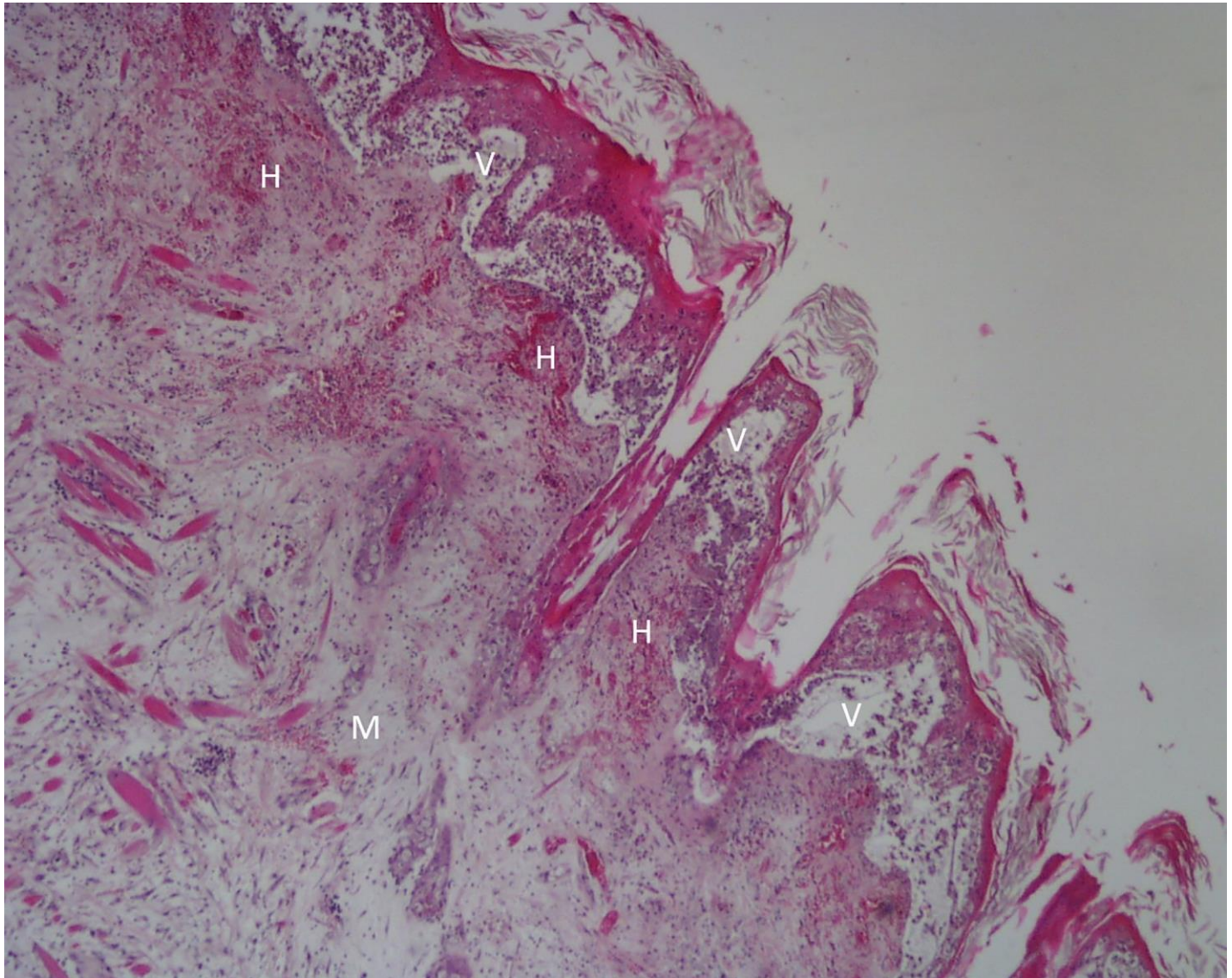
Fig 5. Lip of hare-2. Intraepidermal and subepidermal vesicopustules (V) that appear as "empty spaces" between the dermis and the epidermis, causing the detachment of the epidermis; mixomatous tissue (M) in the dermis characterized by abundant extracellular matrix pulling apart the fibroblasts; microhemorrhages (H) in the uppermost layer of the dermis;. H&E, 40x.

NCBI blast analysis (28.02.2020) of the DNA polymerase nucleotide sequences confirmed homology with herpesvirus *DNA polymerase* coding sequence from other mammals. Though with a low query cover of 48%, 78.31% of similarity was observed with a Phocid herpesvirus 2 (NC_043062.1) and 77.11% with Megabat gammaherpesvirus (LC268920.1) and a Harp seal herpesvirus (KP136799.1).

Blast analysis of the hares' DNA polymerase deduced aa sequences (28.02.2020), showed 52.63% identity over a query cover of 100% with bat herpesviruses (ALH21079.1, ALH21081.1 and ALH21071.1). Similarity was also found with Equid gammaherpesvirus 5 (AAD30141.1) showing 72.50% of identity and 62% of query cover, and with Asinine herpesvirus 4.1 (AAL14768.1), displaying 62% of identity over a query cover of 82%.
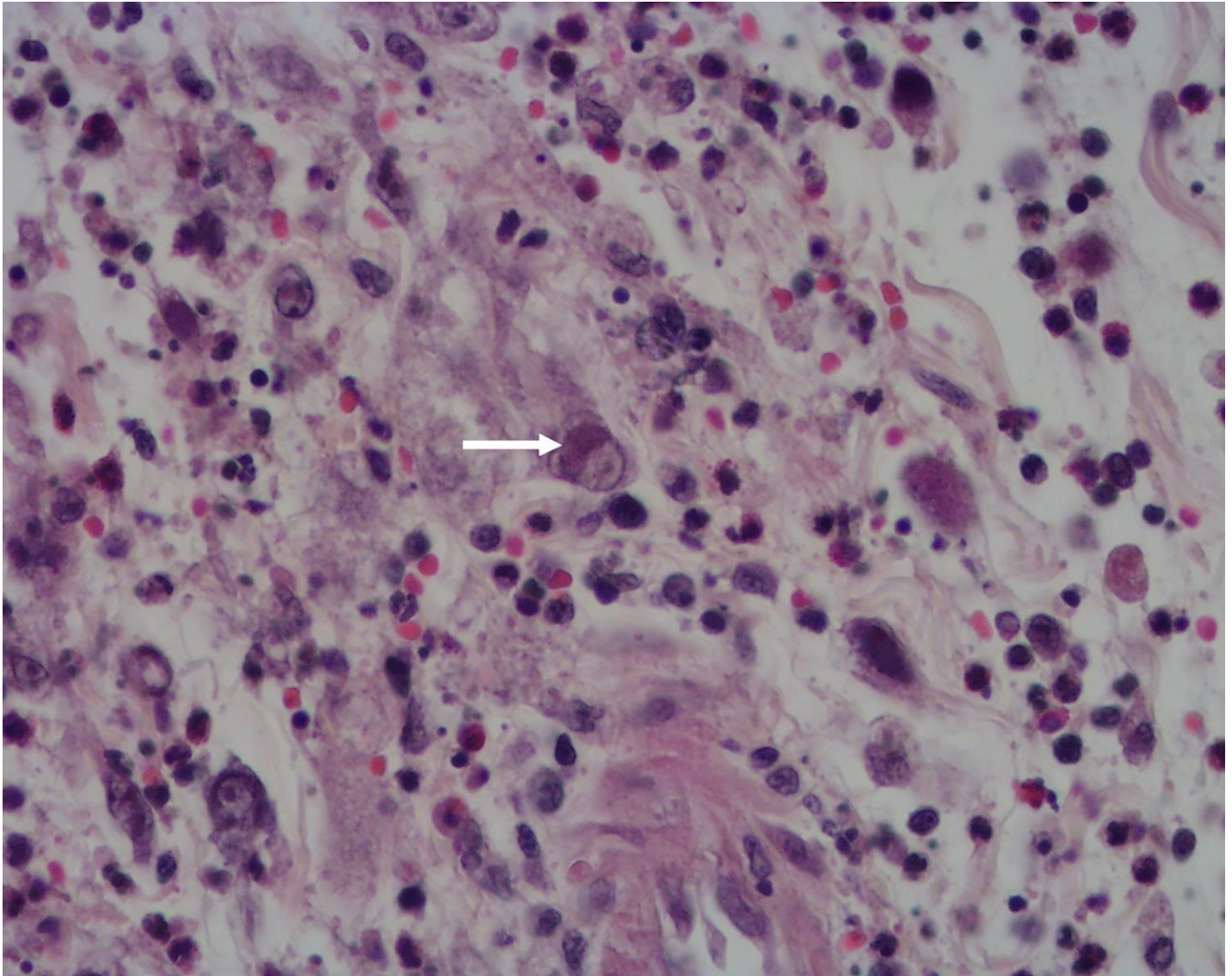
Fig 6. Lip of hare-2. Oedema and inflammatory cell infiltration with signs of necrosis. An intranuclear inclusion body in a mesenchymal cell can be seen (arrow). H&E, 400x.

https://doi.org/10.1371/journal.pone.0231795.g006

An amplicon ~500bp long, was obtained from three DNA polymerase positive hare samples, with the genus-specific glycoprotein B (gB) gene primers described by [17].The three gB consensus sequences (453 bp long) showed 100% similarity to each other. These sequences were submitted to the GenBank (MN557129-31).

Blast analysis (23.03.2020) of the MN557129 sequence confirmed homology with Glycoprotein B sequence of known herpesvirus, namely with wood mouse (*Apodemus sylvaticus*) herpesvirus (GQ169129.1, EF495130.1 and EF128051.2, showing 67.69% to 70.72% similarity with a query cover of 88% to 92%), bank vole (*Myodes glareolus*) rhadinovirus (AY854169.2, 69.56% similarity and 92% query cover), field vole (*Microtus agrestis*) rhadinovirus (EF128052.1,, 67.69% similarity and 92% query cover), and chimpanzee (*Pan troglodytes*) rhadinovirus 1 and 2 (GQ995451.1 and EU085378.1, 65–67% similarity with 97–98% query cover).
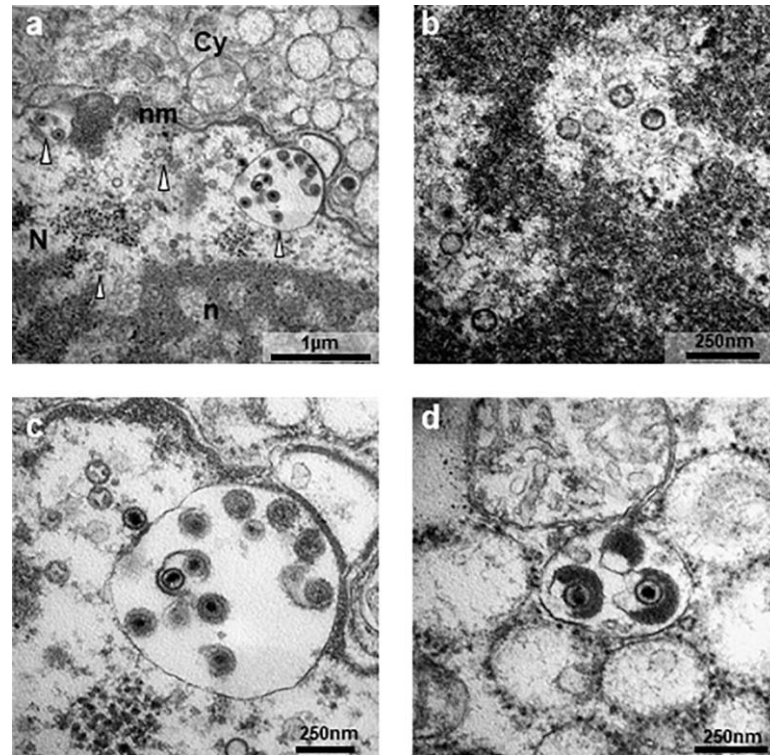
Fig 7. Electron micrographs of penile soft tissue of hare-1. a- Overview of a virus-infected cell (N, nucleus; n, nucleolus; nm, nuclear membrane; Cy, cytoplasm; arrowhead, viral particles); **b-** Naked capsids seen in areas of euchromatin in the nucleolus. **c-** DNA-loaded capsids close to the nuclear membrane in the process of budding into the perinuclear space; **d** Tegument assembly in the cytoplasm of the host cell. Photos obtained in a transmission electron microscope Hitachi H-7000 using iTEM software and Megaview III mid-mounted camera.

https://doi.org/10.1371/journal.pone.0231795.g007

3.6. Phylogenetic analysis

It has been shown that, despite reduced, the region amplified by the nested PCR has discriminatory power to allow phylogenetic inferences [16]. To investigate the phylogenetic relationship of the Iberian hare herpesviruses found in this study (represented by sequence MN557129), with other members of the Herpesviridae family, a set of 37 DNA polymerase protein sequences from alpha-, beta- and gammaherpesviruses, obtained from GenBank, were edited to span a 54 to 59 aa-residue region comprising the homologous regions encoded by sequence MN557129.

Despite many polytomies and low bootstrap values, the analysis of the DNA polymerase protein sequences by unrooted Maximum Likelihood method and LG+G+I model [20] corroborated that the herpesvirus sequence from *Lepus granatensis* grouped within the gammaherpesvirus cluster (Fig 8).

To refine the phylogenetic inference within gammahespesviruses, the variability within the gB protein was explored in a set of 45 gammaherpesviruses using the clades described by [17] as reference. Two trees were constructed, the first based on the partial gB protein sequences alone, and the second based on concatenated DNA polymerase and gB sequences. Concatenation enabled greater phylogenetic resolution. The tree with the highest log likelihood (-3335,05) is shown in Fig 9. The accession numbers of the original sequences from which the DNA Polymerase and Glycoprotein B genes were edited, are indicated in the respective legend (Fig 9).
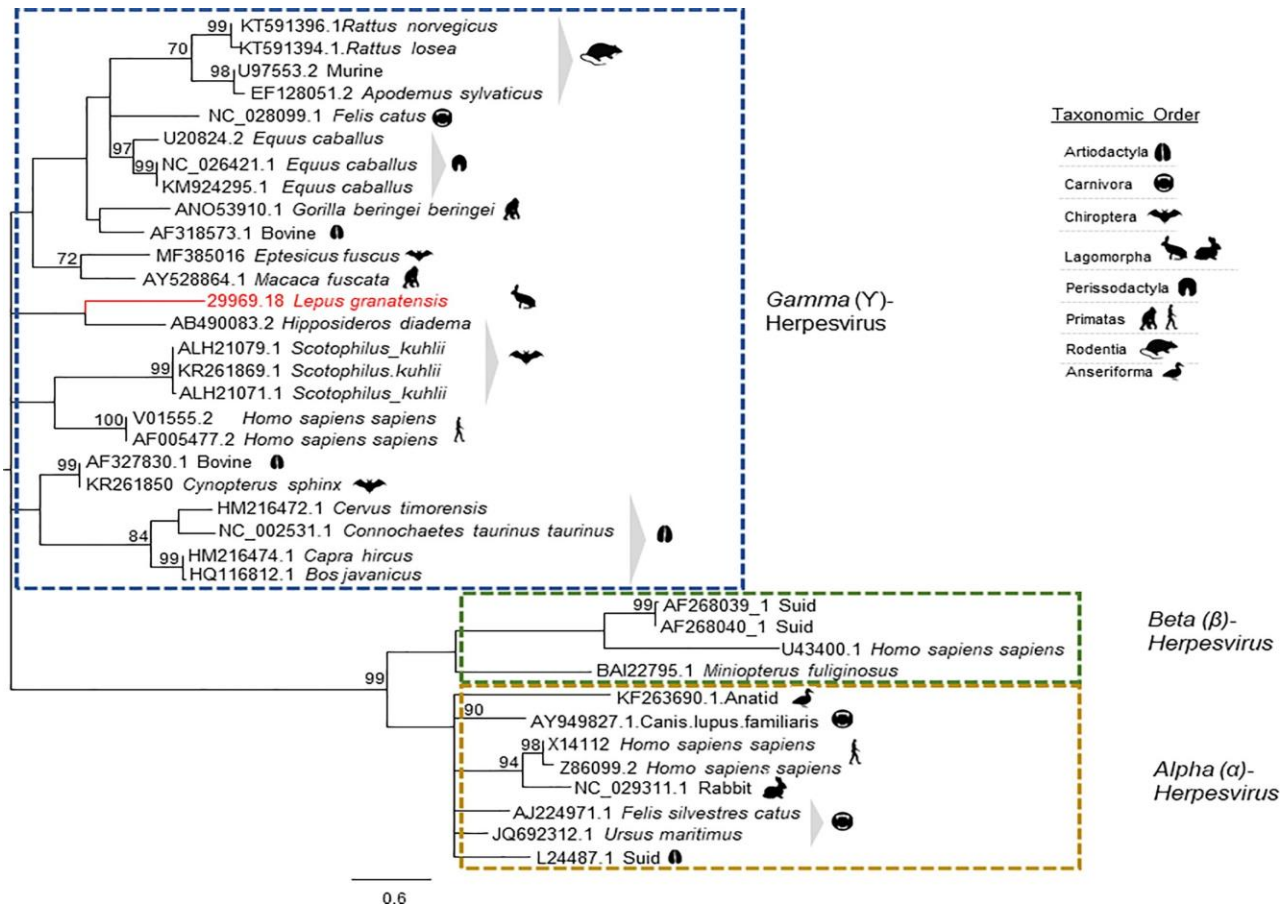
Fig 8. Phylogenetic analysis based on 37 partial *DNA Polymerase* amino acid sequences of herpesviruses from several vertebrate species. The access number of the nucleotide sequences from which the amino acid sequences were deduced are given. The tree with the highest log likelihood (-2432.55) is shown. The LG+G+I model considering 5 categories, [+G] parameter of 0,9929 and [+I] of 11,47% sites was used. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. There were a total of 60 positions in the final dataset. Robustness of the tree nodes was assessed by bootstrapping 1000 times. Only bootstrap values ⟩70 are shown. The evolutionary analyses were conducted in MEGA X [18] and the phylogenetic tree was edited in the Figtree software version 1.4.0.

https://doi.org/10.1371/journal.pone.0231795.g008

This phylogenetic analysis confirmed that the leporid herpesvirus under study is more closely related with gammaherpesviruses from Murine rhadinovirus (MHV-68 and MuHV-4), *Apodemus flavicollis* rhadinovirus (AflaRHV-1), *Apodemus sylvaticus* rhadinovirus 1 (AsylRHV1), *Apodemus sylvaticus* herpesvirus (WMHV), *Bandicota indica* rhadinovirus 4 (BindRHV-4), *Microtus agrestis* rhadinovirus 1 (MagrRHV1) and *Myodes glareolus* rhadinovirus 1 (MglaRHV1), but clearly diverge from this group, forming a separate clade supported by a bootstrap value of 100 ([Fig 9](#)). In this tree, no polytomies were observed.

Despite more information on the genome of this herpesvirus is required, this preliminary analysis suggest that it may represent a specific replicating lineage within the rhadinovirus genus. In accordance, we propose to name this virus Leporid gammaherpesvirus 5 (LeHV-5), following the rabbit alphaherpesvirus 4 (LeHV-4), the only leporid herpesvirus recognised so far as a species by the ICTV.

3.7. Isolation of the viruses in cell cultures

The difficulties found in viral isolation in CRFK, Vero, RK13 and Hella cells may be explained by the fact that LeHV-5 is a gammaherpesvirus.

Despite LeHV-5 seems unable to multiply in human (Hela) and primate (Vero) cells, given the zoonotic potential of some animal herpesviruses, as the case of the cercopithecine alpha-herpesvirus 1 [21] and the murine gamma herpesvirus 68 [22], all isolation attempts were carried out in BSL-2 conditions.

4. Discussion and conclusion

This study describes the detection of the first herpesvirus (Leporid gammaherpesvirus 5) in the genus *Lepus* that, according to the phylogenetic analysis based on DNA polymerase and gB concatenated sequences, is more similar to rodent gammaherpesviruses of the rhadinovirus genus.

Viral isolation was not successful in RK13, CRFK or Vero cells. The difficulty in growing the virus *in vitro*, an important step towards its characterisation, may be a consequence of the co-infection with MYXV. The greater ease in the multiplication of MYXV, which grows in many cell cultures [23,24], may have hampered herpesvirus isolation. Moreover, wild animal samples are frequently somewhat autolysed and usually frozen before reaching the laboratory, which may lead to herpesvirus inactivation. The complexity in isolating genital gammaherpes-virus in cell culture was also referred by [25]. The lack of cell cultures from *Lepus* species may pose further challenges.

During our investigation, we observed necrosis of the genitals and herpetic-like vesicles in the lips of hares co-infected with LeHV-5 and MYXV, which were attributed to herpesvirus. The presence of LeHV-5 in the penile of hare-1 was confirmed by PCR and TEM, as shown in Fig 7. Herpesvirus particles were also visualised in epithelial and stroma cells of the eyelid of hare-2.

Notwithstanding the sampling limitation, the fact that macroscopic lesions were only seen in animals with myxomatosis, suggests that MYXV may play a role on herpesvirus replication and/or reactivation by compromising the immune response of the host, leading to the subsequent development of clinical disease with exuberant lesions. Immunosuppression facilitates herpesvirus infections and virus reactivation and it was demonstrated that certain MYXV proteins, such as Serp-1, have strong immune suppressing effects [24]. MYXV infection may hence represent a stress and/or immunosuppressive triggering factor for herpesvirus infection. It is known that stress, disease and other factors such as extreme temperatures can lead to the resurgence of herpesviruses in other species [26]. It was observed that depressed T-cell immune function reduces the quality of immune surveillance resulting in the increase of viral activity [27].

Herpesviruses generally follow one of three distinct strategies [27] within the host, namely i) latency with occasional re-emergence, ii) hit-and-run' approach and iii) slow-and-low' tactic

[27].In the case of LeHV-5, because it affects wildlife, specimens are mainly animals found dead or moribund, limiting the conclusions on the strategy of the virus.

In addition, herpesviruses are frequently found either in the absence of clinical signs or in association with very diverse clinical signs [28].This fact muddles the understanding of the true role and relative contribution of many herpesviruses in the courses of certain diseases, especially with regards to wild species that are often exposed to, and infected by, many pathogens. On the other hand, animal experimentation is complicated by the absence of available specific pathogen free (SPF) specimens, and by the difficulties in keeping hares in captivity, limiting cause-effect experiments. Moreover, during latency, herpesviruses may not be detected by current methods as it is the case of gammaherpesvirus in horses, resulting in an underestimated prevalence in the populations [28,29]. Reports indicate that equine herpesvirus 2 (EHV-2) can be detected in immunocompetent animals in the absence of signs of disease (revised on [28]) meaning that healthy animals can be a potentially source of viral transmission.

According to our study, based on viral DNA amplification, around half of animals tested (63% symptomatic and 37% asymptomatic) were positive for LeHV-5. However, this value may be an underestimation given that the tropism of LeHV-5 is still unknown, and consequently the tissue samples used for diagnosis may have been inadequate. Thus, we cannot assure that PCR negative animals were not false negatives.

The fact that herpetic lesions were not observed in young, could mean that if the acquisition of LeHV-5 occurs at an early age, primary infection takes place with mild or no symptoms. In the absence of an unbalancing triggering factor such as MYXV infection, LeHV-5 may successfully establish a long-term relationship with the hare host, with subclinical disease and transient viremia. This would explain the detection of herpesvirus DNA in apparently healthy hares.

Interestingly, the gammaherpesvirus identified in external genitalia of the investigated hares was not associated with the development of papillary lesions as in other genital gammaherpesviruses' infections [25]. However, given that hunters, hunting managers and landowners have the opinion that the reproduction of the Iberian hare has been declining in recent years, it is important to clarify if this reduction is also associated with the emergence or circulation of LeHV-5. Other important concern is the potential production of oncogenic proteins by this herpesvirus.

According to our findings, genital herpesvirus may have a critical effect on hares' fertility and reproduction as well as in their survival. Hence, it is crucial to evaluate and understand the extent to which MYXV plays a role in the infection/reactivation of herpesvirus, as well as the putative role of herpesvirus in favouring infection of hares by MYXV or aggravating the severity of myxomatosis clinical forms.

The virological results obtained in this study also disclosed the infection of apparently healthy hares by LeHV-5, suggesting the possible circulation of this virus in the wild populations in a subclinical form. Because herpesvirus-DNA was detected in internal organs (liver and spleen), this asymptomatic infection may be systemic.

Although the Iberian hare populations are still considered stable, no census is available for the populations in Portugal. Presently, we continue monitoring apparently healthy and MYXV-positive hares in mainland Portugal to determine the extent of the geographic distribution of LeHV-5 among the wild hare populations, and the putative association of herpesviruses with the virulence of the recently emerged hare myxoma virus.

It is of paramount importance to evaluate the geographical distribution of the virus in the hare populations, the real extent and severity of the lesions induced in hares by LeHV-5, the persistence and latency of herpesvirus in the wild populations and the LEHV-5-MYXV

associated pathology in order to predict the consequences of the LEHV-5 infection at population level and to evaluate its importance in the future of this iconic species.

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References

1. Soriguer R, Carro F. Lepus granatensis. The IUCN Red List of Threatened Species 2019: e. T41306A2953195. 2019. http://dx.doi.org/10.2305/IUCN.UK

2. Palacios F. On the taxonomic status of the genus Lepus in Spain. Acta Zool. 1983;Fennica 17: 27–30.

3. Fenner F, Fantini B. History of Myxomatosis—an Experiment in Evolution. CABI. Biological Control of Vertebrate Pests. CABI. New York, USA; 1999.

4. Barlow A, Lawrence K, Everest D, Dastjerdi A, Finnegan C, Steinbach F. Confirmation of myxomatosis in a European brown hare in Great Britain. Vet Rec. 2014; 175: 75–76. https://doi.org/10.1136/vr.g4621 PMID: 25034685

5. Collins JJ. Myxomatosis in the common hare. Ir Vet J. 1955; 9, 268.

6. Wibbelt G. Infectious Diseases in European Brown Hare (Lepus europaeus). 2014. https://doi.org/10.2461/wbp.2005.1.11

7. Bocanegra IG, Dalton KP, Go´mez JC. First outbreak of myxomatosis in Iberian hares (Lepus granatensis). 2019; 1–5. https://doi.org/10.1111/tbed.13289 PMID: 31293076

8. OIE. Myxomatosis, Portugal. 2018. Available: https://www.oie.int/wahis_2/public/wahid.php/ Reviewreport/Review?page_refer=MapFullEventReport&reportid=28628

9. Jin L, Lo¨hr C V., Vanarsdall AL, Baker RJ, Moerdyk-Schauwecker M, Levine C, et al. Characterization of a novel alphaherpesvirus associated with fatal infections of domestic rabbits. Virology. 2008; 378: 13–20. https://doi.org/10.1016/j.virol.2008.05.003 PMID: 18554680

10. Barthold SW, Griffey SM, Percy DH. Rabbit. 4 th. Pathology of Laboratory Rodents and Rabbits. 4 th. 2016. p. 258.

11. Brown J C., Newcomb W W. Herpesvirus Capsid Assembly: Insights from Structural Analysis. Curr Opin Virol. 2011; 1: 142–149. https://doi.org/10.1016/j.coviro.2011.06.003 PMID: 21927635

12. Davison AJ, Richard E, Bernhard E, Gary HS, Duncan MJ, Anthony MC, et al. The order of Herpesvirales. Arch Virol. 2009; 154: 171–177. https://doi.org/10.1007/s00705-008-0278-4 PMID: 19066710

13. Sunohara-Neilson JR, Brash M, Carman S, Nagy E´, Turner P V. Experimental infection of New Zealand white rabbits (Oryctolagus cuniculi) with leporid herpesvirus 4. Comp Med. 2013; 63: 422–431. PMID: 24210019

14. David M. Knipe PM. Fields Virology. 6th Editio. In: Williams L, Wilkins, editors. Fields Virology. 6th Editio. Philadelphia, PA, USA; 2013.

15. Graham L, Orenstein JM. Processing tissue and cells for transmission electron microscopy in diagnostic pathology and research. Nat Protoc. 2007; 2: 2439–2450. https://doi.org/10.1038/nprot.2007.304 PMID: 17947985

16. Devanter DRVAN, Warrener P, Bennett L, Schultz ER, Coulter S, Garber RL, et al. Detection and Analysis of Diverse Herpesviral Species by Consensus Primer PCR. 1996; 34: 1666–1671.

17. Ehlers B, Dural G, Yasmum N, Lembo T, de Thoisy B, Ryser-Degiorgis M-P, et al. Novel Mammalian Herpesviruses and Lineages within the Gammaherpesvirinae: Cospeciation and Interspecies Transfer. J Virol. 2008; 82: 3509–3516. https://doi.org/10.1128/JVI.02646-07 PMID: 18216123

18. Kumar S, Stecher G, Li M, Knyaz C, Tamura K. MEGA X: Molecular Evolutionary Genetics Analysis across computing platforms. Molecular Biology and Evolution. 2018.

19. H M., K H., Y T. Dating the human-ape split by a molecular clock of mitochondrial DNA. J Mol Evol. 1985; 22: 160–174. https://doi.org/10.1007/bf02101694 PMID: 3934395

20. Le SQ, Gascuel O. An Improved General Amino Acid Replacement Matrix. Mol Biol Evol. 2008; 25: 1307–1320. https://doi.org/10.1093/molbev/msn067 PMID: 18367465

21. Huff JL, Barry PA. Infection in Humans and Macaques: Potential for Zoonotic Disease. Emerg Infect Dis •. 2003; 9.

22. Hricova´ M, Mistr´ıkova´ J. Murine gammaherpesvirus 68 serum antibodies in general human population. Acta Virol. 2007; 51: 283–287. PMID: 18197737

23. Fenner F, Ratcliffe FN. Myxomatosis. Cambridge Univ Press London, UK. 1965.

24. Spiesschaert B, Mcfadden G, Hermans K, Nauwynck H, Walle GR Van De. The current status and future directions of myxoma virus, a master in immune evasion. Vet Res. 2011; 42: 76. https://doi.org/10.1186/1297-9716-42-76 PMID: 21658227

25. Saliki JT, Cooper EJ, Rotstein DS, Caseltine SL, Pabst D., McLellan WA, et al. A Novel Gammaherpesvirus Associated with Genital Lesions in a Blainville's Beaked Whale (Mesoplodon densirostris). J Wildl Dis. 2006; 42: 142–148. https://doi.org/10.7589/0090-3558-42.1.142 PMID: 16699156

26. Izumi Y, Tsuduku S, Murakami K, Tsuboi T, Konishi M, Haritani M, et al. Characterization of bovine herpesvirus type 4 isolated from cattle with mastitis and subclinical infection by the virus among cattle. J Vet Med Sci. 2006; 68: 189–193. https://doi.org/10.1292/jvms.68.189 PMID: 16520546

27. Grinde B. Herpesviruses: latency and reactivation? viral strategies and host response. J Oral Microbiol. 2013; 5: 125–136. https://doi.org/10.3402/jom.v5i0.22766 PMID: 24167660

28. Marenzoni M, Stefanetti V, Danzetta ML, Timoney PJ. Gammaherpesvirus infections in equids: a review. Vet Med Res Reports. 2015; 91. https://doi.org/10.2147/vmrr.s39473 PMID: 30155436

29. Williams KJ, Robinson NE, Lim A, Brandenberger C, Maes R, Behan A, et al. Experimental Induction of Pulmonary Fibrosis in Horses with the Gammaherpesvirus Equine Herpesvirus 5. PLoS One. 2013; 8: 1–15. https://doi.org/10.1371/journal.pone.0077754 PMID: 24147074

30. Zygraich N, Berge E. Experimental Infection of Rabbits and Monkeys with Herpesvirus cuniculi. 1972; 13: 241–244.

31. Hesselton RM, Yang WC, Medveczky P, Sullivan JL. Pathogenesis of Herpesvirus sylvilagus infection in cottontail rabbits. Am J Pathol. 1988; 133: 639–647. PMID: 2849303

32. Akatsuka K, Nii S. Tubular Structures Detected in the Nuclei of Human Embryonal Lung Fibroblasts Infected with Human Cytomegalovirus Kazuya AKATSUKA and Shiro NII * of Virology, Okayama Okayama (Accepted for publication, December Various viruses with icosahedral capsids. MicrobiolImmunol. 1986; 30: 289–296.

33. Hinze HC. New Member of the Herpesvirus Group Isolated from Wild Cottontail Rabbits. 1971; 3: 350–354.

34. Brash ML, Nagy É, Pei Y, Carman S, Emery S, Smith AE, et al. Acute hemorrhagic and necrotizing pneumonia, splenitis, and dermatitis in a pet rabbit caused by a novel herpesvirus (leporid herpesvirus-4). Can Vet J. 2010; 51: 1383–1386. PMID: 21358932

35. Roizman B, Pellett P. The family Herpesviridae: a brief introduction. 4th ed. In: Knipe DM, Howley P., editors. 4th ed. Philadelphia: Lippincott Williams & Wilkins; 2001. pp. 2381–2397pp.

36. Duarte MD, Carvalho CL, Barros SC, Henriques AM, Ramos F, Fagulha T, et al. A real time Taqman RT-PCR for the detection of rabbit hemorrhagic disease virus 2 (RHDV2). J Virol Methods. 2015; 219: 90–95. https://doi.org/10.1016/j.jviromet.2015.03.017 PMID: 25823548

37. Duarte MD, Barros SC, Henriques AM, Fagulha MT, Ramos F, Luıs T, et al. Development and validation of a real time PCR for the detection of myxoma virus based on the diploid gene M000. 5L / R. J Virol Methods. 2014; 196: 219–224. https://doi.org/10.1016/j.jviromet.2013.11.014 PMID: 24300832

Chapter 5

General Discussion

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This chapter is not intended to repeat the discussions of the articles included in this thesis, but rather to explain how the different studies interrelate and to share the author's personal view towards the various studies.

The genus *Oryctolagus* appeared in the fossil record (Middle Pliocene) before any other modern leporid genus (Villafuerte and Delibes-Mateos 2019). According to reported evidence, the species *Oryctolagus cuniculus* would be present across the Mediterranean area and central Europe in the Late-Pleistocene, becoming confined to its current range after the glacial period in the Early Holocene (Lopez-Martinez 2008).

In 2019, the European rabbit (*Oryctolagus cuniculus* Linnaeus, 1758) was classified, for the first time in history, as "endangered of extinction" (Villafuerte and Delibes-Mateos 2019). Considering the characteristics of the species (easy adaptation to diverse habitats, high fertility and prolificity, etc), the population reduction is very alarming for biodiversity conservation. Like a red flag, the 60-70% decline of populations of European rabbit in the last decade led to a decrease of ~65% and ~45% in Iberian lynx and Spanish Imperial Eagle fecundities respectively (Monterroso et al. 2016). This chain effect had an impact on other predators and prey, given the key role of wild rabbit in many of the trophic chains on the Iberian Peninsula (Carvalho et al. 2020).

In recent years, the wild rabbit and the Iberian hare have been gaining growing interest from the academy, civil society, the environmental and ecological organizations and policy makers. A clear example of these were the *Projets +Coelho*, implemented following the constitution of a working group by Dispatch no. 4757/2017 of 31 May of the Portuguese Ministry of Agriculture, to respond to the effect of rabbit haemorrhagic disease in the rabbit population, the *Mixolepus project* in Spain in response to the emergence of ha-MYXV, or the recently approved *LIFE Iberconejo* with an allocation of around 2 million euros for 3 years. These, and other investments, are fully justified given the importance of the wild rabbit in the Mediterranean ecosystem, where its role is so decisive that some ecologists call the Iberian Peninsula the "rabbit's ecosystem" (Delibes-Mateos et al. 2008).

Although excessive hunting contributes to the decline of leporid populations, it is also true that the hunters, along with rural managers and owners, invest highly in the preservation of these species through the supplementation of food, water and shelter during the summer. All projects that depend on stable populations of prey species, such as the conservation projects of Iberian lynx (Life+IBERLINCE), Black vulture (Parque Natural do Tejo Internacional), Imperial eagle (Parque Natural do Vale do Guadiana and ZPE of Castro Verde) among others, take place in hunting areas where management measures with a positive impact on wild species are instituted.

Hunting in Spain generates at least 6,5 billion euros (around 0.3% of PIB, the equivalent of wine sales), and around 187 000 jobs, 1% of the active population in Spain (Andueza et al. 2018). According to ANPC (National Association of Rural Owners, Portugal), in 2021, hunting activity in Portugal generated 330 million euros, 7 300

permanent jobs, adding up to 108 300 temporary jobs. The hunting sector in Portugal, with the adoption of appropriate policies, has an economic potential in the order of 1.14 billion euros a year.

After several decades of research on wild leporids' viral diseases, there are still no effective measures to control the impact of these diseases that have decimated the populations of leporids in the Iberian Peninsula leading to the critical imbalance of our ecosystems.

Viral diseases have been identified as the main causes of the leporid decline (Duarte et al. 2018; Duarte et al. 2021). The importance and severity of viruses were evidenced during the three years of field work performed within the scope of this doctoral thesis when it was possible to testify to the emergence of myxomatosis in Iberian hare by the natural recombinant ha-MYXV (Carvalho et al. 2020), cases of co-infection by ha-MYXV and classic MYXV in hares and rabbits, never detected before (Abade dos Santos et al. 2022) the spillover of the ha-MYXV (natural recombinant myxoma virus) from hares to rabbits (Abade dos Santos et al. 2020; Abade dos Santos et al. 2020), the identification of a herpesvirus in hares undergoing myxomatosis (Abade dos Santos et al. 2020) not described before, and the detection of an Iberian hare infected with rabbit haemorrhagic disease (Velarde et al. 2021).

Taking into account that the European rabbit was recently classified as an "endangered species" by the IUCN, it seems also inevitable that other leporid species, in particular the Iberian hare, will receive a similar or worst status classification in the next revisions. According to the Living Planet Report 2020 from World Wildlife Fund (WWF), the global wildlife populations have declined by an average of 60% over the past 40 years, demonstrating that the planetary biodiversity is threatened (WWF 2020).

This thesis aimed to study the current major viral threats to the Iberian Leporids conservation, in particular to the wild leporids from Portugal, the *Oryctolagus cuniculus algirus* and the *Lepus granatensis* and to contribute with practical measures to help disease control and population recovery. The study took into account the co-existence in *O. c. algirus* and *L. granatensis* of MYXV and RHDV2 in space and time, and the fact that both species are affected by related viruses. In particular, we aimed to extend our knowledge of three viral diseases, by studying their epidemiology, pathophysiology and phylogenetics, having had the opportunity to report the emergence of new viruses and viral strains. The work also focused on prophylaxis and the development of versatile, specific and highly sensitive diagnostic methods to assist in the monitoring and control of diseases. Although each disease and related studies are presented in a different chapter, they intersect each other as shown by the co-infection by MYXV and RHDV (Chapter 3, scientific publication 8) and by MYXV and Leporid gammaherpesvirus 5

reports (Chapter 4, scientific publication 14).

In general, the present studies favoured applied research by directly approaching real problems of the rabbit industry or the concerns related to the conservation of the wild rabbit and Iberian hare. Examples of this were the development of the first method for molecular differentiation of myxoma viruses (Chapter 2, scientific publication 4), a relevant tool to assist the selection of sanitary prophylaxis strategies in the industry and to support conservation studies, and the first method for the molecular diagnosis of LeHV-5 (Chapter 2, Scientific publication 5), whose impact and relevance is still obscure. Another important achievement was the confirmation of the susceptibility of wild rabbits and domestic rabbits to the naturally recombinant strain of MYXV (ha-MYXV), isolated from rabbits and Iberian hares (Chapter 4, scientific publications 10, 11 and 13).

The efficacy of the protection induced by commercial myxomatosis vaccines against this new myxoma virus was also investigated, allowing us to confirm that vaccines can induce protection of the Iberian hare against ha-MYXV infection using a higher titre of heterologous commercial vaccine virus (Chapter 4, scientific publication 13). This last result is particularly important for the conservation of the Iberian hare in genetic reserves and breeding centres, while a specific vaccine is not available.

The scientific work developed required the combination of several areas of knowledge and complementary skills. For example, the commercial vaccine evaluation trial (Chapter 4, scientific publication 13) required the capture of the animals in the field, a demanding and extremely laborious procedure that involved hundreds of volunteers. Hares were kept in specifically designed and built cages, and the animal experimentation was conducted in a BSL-2 mobile unit designed and constructed for the sole purpose of this study. The experimental study led to the implementation of the first captive breeding centre of the Iberian hare in Portugal. Blood was drawn from wild animals using a technique validated during this PhD (Chapter 2, Scientific publication 1). Several molecular methods namely the MYXV multiplex, the IFT and the seroneutralization test were designed, validated and applied. Finally, it is expected that all the knowledge and tools produced during this thesis will benefit the Iberian Hare Reproduction Center (*www.lebre-iberica.pt*).

The broad range of techniques used in the investigations carried out for the development of this thesis, made possible in-depth studies of various topics, and the familiarization and understanding of the fundaments and potentialities of many laboratory techniques, namely conventional PCR, real-time PCR, PCR with reserve transcription, primer and probe design, melting analysis design, bacterial cloning, Sanger and Next Generation genomic sequencing, nucleotide sequence alignment, gene annotation, nucleotide and amino acid phylogeny, serology, production of primary cells and cell-line

multiplication, virus isolation and purification, virus titration, necropsy and histopathological analysis, immunohistochemistry, immunofluorescence, electron microscopy, animal experimentation, among others.

The need to adapt the technique of collecting blood from the external jugular vein in leporids resulted from the regular need to collect a relatively high volume of blood in these small animals, which have very small and difficult to visualize veins, and that are very sensitive to manipulation and containment. This is particularly evident when it is necessary to collect about 1 ml from a 250 g juvenile wild rabbit specimen. For this reason, the external jugular vein route was evaluated, proving to be easy, very safe and well accepted by the animals. This technique was applied in all blood collection processes carried out within the scope of the investigations for this doctoral dissertation. Bearing in mind that animals are often returned to their natural environment immediately after the procedure, a camera trapping assessment confirmed their well-being shortly after the procedure. Although there are other alternatives for blood collection, this has shown, throughout the experiments described in the article and throughout the experience accumulated in subsequent experiments, to have excellent efficacy, avoiding long-term handling of the animal, bruises formation and repetition of the procedure due to insufficient collection or unsuccessful puncture (Abade dos Santos et al. 2019).

Blood is the most informative biological material that can be collected from an animal. However, the analysis of biological matrices to the detriment of the animal evaluation itself creates a gap between the sample itself and the pathophysiological process of the disease, which should be the main focus of attention, particularly in the animal health field. Molecular and cellular biology nowadays provides us with key and indispensable tools for the study of pathological agents and diseases. However, it is necessary to frame the results obtained with these powerful techniques with the pathophysiological process itself, questioning whether or not the detected organisms exist in the host and were related to disease, as reviewed and discussed in Chapter 2, Scientific publication 3 (Abade dos Santos et al. 2021).

Molecular based detection methods have some unquestionable advantages compared to the methods mentioned above, including their greater sensitivity, specificity and ability to be automated. However, the component that is being detected, whether that be genomic components or messenger RNA, is paramount to the interpretation of a positive result. Therefore, the use of sensitive laboratory techniques to test for the presence of novel viruses must be supported by additional clinical evidence to convincingly indicate that the detected virus was the cause of the observed disease (Griffiths 1999). For example, the recent COVID-19 pandemic has highlighted the importance of correct interpretation of molecular biology test results given its potential to influence global

guidance on appropriate time of patient discharge and isolation length.

This precaution is particularly important when reporting new pathogens, as is the case with the LeHV-5 also described in this thesis (Abade dos Santos et al. 2020), for which we do not yet know its real importance as a pathogenic agent, taking into account the still scant knowledge on the role of viruses as commensal agents or even as agents useful to the host they infect or simply colonize.

Regarding animal health, there is a large gap between the information available at the diagnostic laboratories and the information in the field. Laboratories search for a specific set of etiological agents requested by the veterinarian, who is often not informed about the new emerging or re-emerging organisms, given the delay between scientific data being reported in scientific journals and its delivery to clinicians through specialized newspapers in veterinary medicine. Also, often associated with the etiological agent responsible for the most evident clinical signs exhibited by the animal, other insidious or even uncharacterized etiological agents may interfere, contributing to the pathological process itself. In the end, if there is no scientific interest beyond what is required for diagnosis, these agents may remain unknown or uncharacterized. For example, it would have been difficult for the new natural recombinant RHDV to be identified in domestic rabbits (Abade dos Santos et al. 2021) if a project such as Project +Coelho had not been approved.

Very practical examples of the need for continuous animal health research projects are also reported in this document, such as the discovery of cases of co-infection by myxoma virus and RHDV2 (Chapter 3, Scientific publication 8) (Carvalho et al. 2020), described for the first time, as well as the discovery of LeHV-5 (Abade dos Santos et al. 2020), the first herpesvirus reported in the Iberian hare. Another example is the simultaneous infection by two strains of myxoma virus (classic MYXV and ha-MYXV) in hares and rabbits. In fact, before this report, the classic MYXV virus had not been found in the Iberian hare.

The molecular detection, differentiation and study of these and other diseases, namely viral aetiology, depend upon updated knowledge of their evolution and variability, the prediction of potential mutations and the development of sensitive and easy to perform techniques for rapid diagnosis and quantification, such as those carried out at the Laboratory of National Reference for Animal Health in Portugal (INIAV, IP) for the detection of RHDV2 (Duarte et al. 2015) or the detection of MYXV, less prone to mutations (Duarte et al. 2014).

During this doctoral work, several diagnostic techniques were also developed, namely the first specific method for the molecular diagnosis of LeHV-5, using a method adaptable to Evagreen or hydrolysis probes (Abade dos Santos et al. 2021), a

quadruplex real-time PCR (also adaptable to Evagreen system) to virtual detect and distinguish all myxoma strains including classical type strains and the ha-MYXV type strains (Abade dos Santos et al. 2021). Also, a high-resolution melting technique was developed capable of detecting a single nucleotide (cytosine) deletion in the M152R gene, found in MYXV-type strains which seems to be associated with virulence variation. These powerful diagnostic methods allowed us to detect the first cases of co-infection by MYXV and ha-MYXV in hare and wild rabbit, exposing another threat to the fragile state of functional extinction that these species face. The detection of this co-infection in Iberian hare also justifies the need for further studies on the mechanisms of susceptibility of the species to myxoma viruses, as there is evidence of some susceptibility of hares to classical myxomatosis viruses.

The LeHV-5/18S rRNA gene duplex system developed and validated (described in Chapter 2, Scientific publication 5), allows for the quantification of this virus in different tissue samples, and provides a diagnostic tool to perceive its tropism and physiopathology. This method constitutes the first rapid and quantitative diagnosis method for leporid gammaherpesvirus 5 (LeHV-5). Considering that the virus was reported for the first time in 2018, retrospective and prospective studies are important to elucidate the extension of the virus' geographical spread in the hare populations.

In the same Chapter, the Scientific publication 4 describes the first real-time method to detect ha-MYXV strains that simultaneously enables investigating the presence of classic MYXV strains and putative co-infections. The three PCR systems that are integrated into the mPCR (directed to M000.5 L/R, M009L and M060L genes) are validated by the amplification of internal control (18S rDNA), previously demonstrated to be a strong and stable housekeeping gene (Abade dos Santos et al., 2021). The sensitivity and specificity of these PCR systems were of 100% when samples previously classified as positive or negative were screened by the M000.5 L/R-based system (M.D. Duarte et al. 2014) and further classified as recombinant/classic MYXV by the system developed by (Dalton et al. 2019). When the four systems were used together in the quadruplex, the mPCR proved to be robust, highly sensitive and highly specific.

As mentioned above, there is a gap between the production of scientific knowledge and the updating of information to clinical practicians. Viral Hemorrhagic disease provides a good example of this. One year after the emergence of RHDV2 (2010), it was already known that the immunity conferred by RHDV1/a was not protective for the emerging RHDV2 (Le Gall-Reculé et al. 2013). Despite this, it was only in September 2016, that the first liver-derived vaccine against the new variant RHDV-2 was introduced into the European market (Eravac, Laboratorios Hipra S.A., E), followed by a second one in March 2017 (Filavac VHD K C+V, FILAVIE, F Roussay). However, the

introduction of these new vaccines in the market occurred without the removal from the market of all vaccines against RDHV, leading to their continued administration, namely in Portugal.

Until 2019, Myxo-RHD was the only vaccine available in a single dose for pet rabbits. In November 2019, the first vaccine against RHDV2 for companion rabbits (Myxo-RHD PLUS) was authorized. This reality has led to mortality in industrial cuniculture, domestic rabbitries and companion rabbits whose value is unknown, but which is certainly large, considering the known dissemination and number of outbreaks registered in wild species. A very practical case is highlighted in Chapter 3, Scientific publication 7, in which a case of an atypical clinical course (subacute or chronic) of RHDV2 in a pet rabbit probably resulted from insufficient immunity conferred by the vaccine against RHDV. The clinical signs and the results of the diagnostic investigations (ultrasound hepatic and peri-hepatic changes, an elevated marker of liver injury - ALT, raised total bilirubin and jaundice, hyperglobulinemia and poor coagulation (namely severe subcutaneous haemorrhage and increased prothrombin time (PT) and activated partial thromboplastin time (aPTT)) were all compatible with Rabbit Haemorrhagic Disease. The atypical development of the clinical course, much longer than the usual, could have resulted from deficient cross-immunity conferred by RHDV vaccination, leading to a proctated disease.

Although RHDV vaccines do not confer full protection against RHDV2, rabbits vaccinated with RDHV cannot be assumed to be immunologically naive against RHDV2 as non-vaccinated animals. The cross-immunity between RHDV and RHDV2 is reported as deficient but not as inexistent (Le Gall-Reculé et al. 2013). Therefore, it is expected that RHDV-vaccinated animals do not show a primary immune response to RHDV2 infection, since cross-reactive responses (RHDV-RHDV2) to particular epitopes may be beneficial to the protective response, even if deficient.

After its emergence, RHDV2 strains evolved quickly, with some variation of amino acids in the capsid protein but maintaining the original RHDV2 antigenic profile. Like other RNA viruses, RHDV presents high recombination rates, the key role of recombination in the evolution of GI.2. is well-documented with several intergenotypic and intergenogroup recombination events, namely between pathogenic and benign viruses. The most frequently identified recombination breakpoints are situated at the RdRp/VP60 boundary or in the junction between p16 and p23 genes (Lopes et al. 2015; Hall et al. 2018; Silvério et al. 2018; Abrantes et al. 2020; Szillat et al. 2020).

Several RHDV2 recombinants containing the non-structural protein genes of other rabbit lagoviruses (such as GI.1b (G1) and GI.4 or GI.3 (NP-CV)) have been identified in

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Europe (Silvério et al. 2018).These include the structural protein (VP60 and VP10) encoding genes of RHDV2 combined with the non-structural protein encoding genes of GI.1b (RHDV genogroup G1 strain), non-pathogenic rabbit caliciviruses Australia 1 (RCV-A1)-like viruses (GI.4) or other non-pathogenic lagoviruses (GI.3) (Dalton et al. 2018; Lopes et al. 2018; Silvério et al. 2018).

These recombinant strains are currently so frequent that were detected in the dwarf rabbit reported in this document (Chapter 3, Scientific publication 7) (Abade dos Santos et al. 2021) as well as in the Eurasian badgers (Chapter 3, Scientific publication 6) (Abade dos Santos et al. 2021), which strains were characterized as GI.4P-GI.2 (Abade dos Santos et al. 2021). The Eurasian badger was identified for the first time as a species susceptible to RHDV2 (Abade dos Santos et al. 2021), associated to a chronic disease evolution. Excretion of the virus through fecal material may serve as a virus spreader among badger sympatric species, namely the highly susceptible wild rabbit, the Iberian hare (despite only one specimen being reported infected), as well as other micromammals in which the virus (RHDV, sensu latu) has been found, namely the Apodemus sylvaticus, *Mus spretus* (Merchán et al. 2011), the *Microtus duodecimcostatus* and the *Crocidura russula* (Calvete et al. 2019).

Due to its genomic characteristics (dsDNA), myxoma virus is less prone to large mutations, namely those that cause the species leap, as in the case of RHDV2. In fact, since its introduction in Europe, in 1952, it has never been found with relevance in any species other than the rabbit, except for a few sporadic cases in the European brown hare (*Lepus europaeus*) and mountain hare (*Lepus timidus*) (Bull and Dickinson 1937; Fenner and Ratcliffe 1965; Micozzi and Palarchi 1965; Barlow et al. 2014).

However, in 2018, a recombinant myxomatosis virus emerged, containing, among other mutations, an insertion of about 2.8 Kbp, capable of inducing the leap from rabbit to Iberian hare, leading to high mortality in this species. For the first time in history, the virus was detected in hares, initially in Spain (Dalton et al. 2019; García-Bocanegra et al. 2019) and then in Portugal (Chapter 4, Scientific publication 9) (Carvalho et al. 2020). Later, this virus was also found in wild rabbits (Chapter 4, Scientific publication 10) (Abade dos Santos et al. 2020) and in domestic rabbits (Chapter 4, Scientific publication 11) (Abade dos Santos et al. 2020) and, as mentioned before, simultaneously along with classical strains in these two species of rabbits.

In the Scientific publication 10, we described three rabbits that died from ha-MYXV with high viral loads found in several tissues. The good body condition suggested a short course of disease. The lower adaptation of the recombinant MYXV strains (ha-MYXV) to rabbits, comparing the MYXV classic strains with which rabbits have evolved for more than 50 years (Kerr et al. 2015), may eventually account for these apparent differences.

Rabbits usually dye from classic MYXV presenting severe swelling of the eyelids and genitals, often accompanied by ocular purulent discharge and very frequently in a state of thinness or cachexia.

Sequencing of the 2.8 Kb insert from the two rabbits showed that both recombinant MYXV strains had the same poxvirus gene "cassette" previously described in Iberian hares (Dalton et al. 2019; Pinto et al. 2019; Carvalho et al. 2020) However, we described a putative truncated gene similar to M066R gene of myxoma virus that is also present, though not annotated, in the myxoma virus sequences obtained previously from Iberian hare. As in the MYXV-Tol (MK836424) and ha-MYXV genomes (MK340973), M062R and the M063R are not found in the insert.

The origin of this insert was discussed previously by other authors and is not a goal of this work. However, the putative protein encoded by ORF M066L is 65.22% to 76.81% similar to homologous ORFs in capripoxviruses, cervidpoxviruses, suipoxviruses, yatapoxviruses but not with BeAn 58058 virus, appointed previously (Pinto et al. 2019) as potential virus donor, or sharing an ancestral donor, of the genetic material found in the insert. On the other hand, the higher similarity of M066L with rabbit fibroma virus and with classical myxoma virus strains, suggests that the insert may have originated from one of these viruses, or a similar virus, not yet described.

The detection of the recombinant MYXV in wild rabbits raises serious concerns at different levels, constituting an additional threat to the already fragile wild rabbit, which entered the IUCN's endangered conservation status last year (Villafuerte and Delibes-Mateos 2019). If the recombinant MYXV and classical MYXV strains behave as different viruses in rabbit, with no full cross-protection between the two, the jump of a recombinant MYXV into the rabbit populations will eventually accelerate the decline of these already diminished wild populations. On the other hand, the fact that the recombinant MYXV affects both the Iberian hare and the wild rabbit, may favour the maintenance of the virus as more hosts are available for virus replication and circulation. The recombinant MYXV may therefore become endemic in the same way that classic strains did, allowing the co-evolution in both species.

From the introduction of myxomatosis in Europe around the 1950s until the development of vaccines, millions of domestic rabbits have died resulting in incalculable economic losses. In Great Britain alone, tens of millions of rabbits have died (Bartrip 2008). Spillover between the wild and domestic animals has been pointed out as the source of infection for outbreaks in the industry (Carvalho et al. 2017).

While studying this recombinant myxoma virus in hare, necropsy and histopathology data showed that another viral agent was responsible for some of the lesions found, namely for the necrotic lesions of the mucosae and cutaneous vesicles

and vesiculopustules. These findings lead us to detect the first herpesvirus in the genus *Lepus*, to which all the hares in the world belong to. This herpesvirus (LeHV-5) was characterized as a *gammaherpesvirus*, subfamily of *herpesviruses*, recognized for its pathologic potential associated with the formation of neoplasms (e.g. lymphomas) and lymphoproliferative diseases (Cesarman 2011).

LeHV-5, which current prevalence and pathophysiology are still poorly known, can now be easily identified and quantified by a qPCR method developed by our team (Abade Dos Santos et al. 2021). This sensitive diagnostic tool allowed us to confirm the isolation of LeHV-5 in several cell lines, namely A20 (*Mus musculus*, B lymphocyte) and BJAB (*Homo sapiens sapiens*, B lymphocyte), after verifying that other cell lines from rabbit and hare, such as RK13 (*Oryctolagus cuniculus*) and HNR (*Lepus europaeus*) were not susceptible to LeHV-5 infection. Virus isolation is a very big step towards the future study of this virus since it will facilitate sequencing of the complete genome and the study of its pathophysiology in murine models, among others.

The concerns about the current state of the Leporidae in the Iberian Peninsula are enormous since I believe that if this population continues to decline, we will be very soon facing the functional extinction of the two species, if not already present. In just three years (the duration of this doctorate), we have witnessed and confirmed several threats that have created or worsened the negative impacts on these species. Studying these wild leporids, which are not very visible by society, proves to be very laborious and complex, although their key role in the ecosystems is largely recognized. Although the fundamental investigation of diseases and the development of diagnostic techniques are of utmost importance, problem-solving based approaches toward the real risks that these species are facing, are still very limited. Today, hunting management by instructed and conscientious hunters are pivotal to the maintenance of the fragile stability of these species. It is therefore crucial that universities and research institutions establish protocols that promote the transfer of knowledge to the field.

An example of a practical approach was the experimental evaluation carried out with commercial myxomatosis vaccines against infection by the new naturally recombinant virus (ha-MYXV) (Chapter 4, Scientific publication 13). This work, which involved many hunters, schools and field agents, and therefore the civil society in numbers approaching 500 individuals, led to the generation of important practical knowledge. Thanks to this study, we know that the currently available commercial vaccines protect rabbits against the new recombinant virus, but we also know how to protect the Iberian hare in genetic reserves by applying these tools.

The commercial myxomatosis vaccines contain live attenuated virus because multiplication of the virus, despite being limited, is important for the induction of a robust

immune response, comprising also cellular immunity which is key for myxomatosis protection (Marlier 2010). As commercial vaccines against myxomatosis contain classic MYXV strains (e.g. VMI 30 strain) or a Shope fibroma virus strain, which are different from ha-MYXV to which the Iberian hare is highly susceptible, low efficacy of commercial rabbit vaccines is expected, given the absence of vaccine virus multiplication in hare cell cultures.

Myxomas (tumour-like lesions) were neither found in the skin of hares or rabbits used in the three studies (Chapter 4, Scientific publication 13) nor in wild rabbits naturally infected with ha-MYXV (Abade dos Santos et al. 2020) although they have already been found in domestic rabbits infected with ha-MYXV (Abade dos Santos et al. 2020). Furthermore, myxomas are not always present in wild hares found dead with ha-MYXV infection, being only present in around 30% of the diseased hares (Abade dos Santos et al. 2020; Abade dos Santos et al. 2020; García-Bocanegra et al. 2020)

According to classic myxoma virus virulence grade classification (Fenner and Marshall 1957; Fenner and Ratcliffe 1965; Fenner and Woodroofe 1965), mortality of 100% in seronegative hares would correspond to Grade I viruses. Additionally, an important difference to highlight from our experimental study is the higher viral load (10-100 times higher titres) in the lungs of rabbits compared to hares, which may be related to the fact that the clinical course in rabbits was more acute. The rabbit proved to be susceptible to ha-MYXV isolated from both wild rabbit and Iberian hare, indicating that wild rabbits may contribute to the spread of ha-MYXV in hares. As two commercial myxoma vaccines (Mixohipra-FSA and Mixohipra-H) showed no efficacy in hares when using commercially recommended dosages, it is urgent to develop a robust vaccine for the Iberian hare or to investigate vaccine efficacy in hares of other commercial myxoma vaccines.

As a common denominator, the outcome from the different studies carried out provides pieces of knowledge about these three viral diseases that upgraded the understanding of the pathogenicity, transmission and evolution of these viruses. This knowledge is useful for various purposes, namely in domestic, livestock and wildlife health, in the hunting sector and on behalf of conservation and biodiversity preservation. In short, this document contains various cross-sectional studies that sought to establish new bridges between the former knowledge and the design of applied solutions for the future control of several current sanitary threats to leporids, key species of our ecosystem.

Chapter 6

Conclusions

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1) Given the frequent/recurrent emergence of new leporid viruses (such as recombinant viruses with the ability to jump through the species barrier or of exhibiting different pathophysiological characteristics), fundamental and applied investigations towards the evaluation of the sanitary condition of the wild populations should be continuous and integrated. In this context, it is particularly important to assess the pathological potential of the new pathogens, particularly when describing them for the first time, by coordinating and integrating molecular biology data, classical virology data and clinical and anatomopathological data to fully disclose the impact of the etiologic agent.

2) To provide robust, quantitative, fast and cheap laboratory diagnoses able to identify the emergence of new virus mutations, it is important to continually redesign and update their molecular compounds to guarantee high sensitivity.

3) The real-time PCR method developed for LeHV-5 allows its laboratory diagnosis and viral quantification in different tissues.

4) The real-time PCR method developed for MYXV/ha-MYXV differentiation allows the diagnosis and quantification of MYXV and ha-MYXV in hares and rabbits. It also allows the detection of co-infections by two strains of MYXV (classical MYXV and ha-MYXV), as found by us in one Iberian hare and one wild rabbit.

5) Although rare or low frequency, RHDV can be found in co-infection with MYXV, aggravating the pathological condition and clinical situation of the rabbit.

6) Genotyping of RHDV, given its high mutation rate, is particularly important in cases of protection failure by commercial vaccines either in the rabbit industry or in pet rabbits. Considering the endemicity of RHDV, it is important that the vaccines on the market, as well as the knowledge of veterinarians, are aligned with the latest scientific knowledge. For this dissemination and knowledge transfer is paramount.

7) RHDV2 has been broadening its range of susceptible hosts, consequently being detected in new geographic regions. This aspect also reinforces the need for continuous monitoring of the virus to predict its phylogeographic evolution. RHDV2 was detected in several Eurasian badgers (*Meles meles*), proving the susceptibility and potential capacity to act as a reservoir host.

8) After 70 years of myxomatosis in the wild rabbit, the first outbreaks of this disease in the Iberian hare appeared in Portugal and Spain in 2018, caused by a naturally recombinant virus (called ha-MYXV, with insertion of 2.8 kbp disrupting the M009L gene), that was identified as the etiological agent.

9) In the first years after its emergence in the Iberian hare, the recombinant ha-MYXV was only identified in this species. Later on, in 2020 however, it was identified as pathogenic for the wild and domestic rabbit, demonstrating some anatomopathological

differences comparing with the Iberian hare.

10) Commercial MYXV vaccines for rabbits have shown total ineffectiveness in the Iberian hare. There is potential for protection after 10-fold increase of the virus titer in the vaccine.

11) The first herpesvirus identified in the genus *Lepus*, called Leporid gammaherpesvirus 5, causes severe pathology in the Iberian hare, affecting the skin and genitals, particularly in hares co-infected with ha-MYXV.

12) In Portugal, the impact of viral diseases on wild Leporidae has been extensively investigated in recent decades, with emphasis on myxoma virus and viral hemorrhagic disease viruses (RHDV, RHDV2). Although the impact of diseases on the wild population has been devastating, leading to over 70% reductions in wild populations, their continuous effect threatens problematically the conservation status of the two species of wild leporids in Portugal.

13) In early 2022, wild leporids, as key species of the Mediterranean ecosystems, deserve further and special attention regarding their health and conservation status.

Chapter 7

References

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This Chapter contains the references of the general Introduction (Chapter 1) and the general discussion (Chapter 5) that do not appear in the references of the various publications included in this thesis.

Abade dos Santos FA, Carvalho Carina, Monteiro M, Carvalho P, Mendonça P, Peleteiro MC, Duarte MD. 2020. Recombinant myxoma virus infection associated with high mortality in rabbit farming (Oryctolagus cuniculus). Transbound Emerg Dis.(August):1–6. doi:10.1111/tbed.13899.

Abade dos Santos FA, Carvalho Carvalho, Pinto A, Rai R, Monteiro M, Carvalho P, Mendonça P, Peleteiro MC, Parra F, Duarte MD. 2020. Detection of Recombinant Hare Myxoma Virus in Wild Rabbits (Oryctolagus cuniculus algirus). Viruses. 12(1127):1–12. doi:10.3390/v12101127.

Abade dos Santos FA. 2018. Quadro anatomo-histopatológico e diagnóstico molecular da doença hemorrágica viral em coelho-bravo. Faculty of Veterinary Medicine, University of Lisbon.

Abade dos Santos F.A., Carvalho CL, Parra F, Dalton KP, Peleteiro MC. 2021. A Quadruplex qPCR for Detection and Differentiation of Classic and Natural Recombinant Myxoma Virus Strains of Leporids. Int J Mol Sci.:1–19.

Abade dos Santos FA, Carvalho CL, Peleteiro MC, Gabriel S, Patrício R, Abade FA, Carvalho CL, Peleteiro MC, Gabriel SI, Patrício R, et al. 2019. Blood collection from the external jugular vein of Oryctolagus cuniculus algirus sedated with midazolam : live sampling of a subspecies at risk Blood collection from the external jugular vein of Oryctolagus cuniculus algirus sedated with midazolam : live. Wildlife Biol. 1(1). doi:10.2981/wlb.00588.

Abade Dos Santos FA, Carvalho CL, Peleteiro MC, Parra F, Duarte MD. 2021. A Versatile qPCR for Diagnosis of Leporid Gammaherpesvirus 5 Using Evagreen® or Taqman® Technologies. Viruses. 13(4). doi:10.3390/v13040715. https://www.mdpi.com/1999-4915/13/4/715.

Abade dos Santos FA, Dalton KP, Casero M, Álvarez ÁL, Parra F, Carvalho CL, Duarte MD. 2022. Co-infection by classic MYXV and ha-MYXV in Iberian hare (Lepus granatensis) and European wild rabbit (Oryctolagus cuniculus algirus). Transbound Emerg Dis.(March):1–7. doi:10.1111/tbed.14540.

Abade dos Santos F A, Magro C, Carvalho CL, Ruivo P, Duarte MD, Peleteiro MC. 2021. A Potential Atypical Case of Rabbit Haemorrhagic Disease in a Dwarf Rabbit. Animals. 11(40). doi:https://doi.org/10.3390/ani11010040.

Abade dos Santos FA, Monteiro M, Pinto A, Carvalho CL, Peleteiro MC, Carvalho P, Mendonça P, Carvalho T, Duarte MD. 2020. First description of a herpesvirus infection in genus Lepus. PLoS One. 15(4):e0231795–e0231795. doi:10.1371/journal.pone.0231795. https://pubmed.ncbi.nlm.nih.gov/32302375.

Abade dos Santos F.A., Pinto A, Burgoyne T, Dalton KP, Carvalho CL, Ramilo DW, Carneiro C, Carvalho T, Peleteiro MC, Parra F, et al. 2021. Spillover events of Rabbit Hemorrhagic Disease Virus 2 (recombinant GI.4P-GI.2) from Lagomorpha to Eurasian badger. Transbound Emerg Dis.(October 2020):1–16. doi:10.1111/tbed.14059.

Abade dos Santos F.A., Portela SJ, Nogueira T, Carvalho CL, de Sousa R, Duarte MD. 2021. Harmless or Threatening? Interpreting the Results of Molecular Diagnosis in the Context of Virus-Host Relationships. Front Microbiol. 12. doi:10.3389/fmicb.2021.647730.

Abrantes J, Droillard C, Lopes AM, Lemaitre E, Lucas P, Blanchard Y, Marchandeau S, Esteves PJ, Le Gall-Reculé G. 2020. Recombination at the emergence of the pathogenic rabbit haemorrhagic disease virus Lagovirus europaeus/GI.2. Sci Rep. 10(1):1–12. doi:10.1038/s41598-020-71303-4. https://doi.org/10.1038/s41598-020-71303-4.

Abrantes J, van der Loo W, Le Pendu J, Esteves PJ. 2012. Rabbit haemorrhagic disease (RHD) and rabbit haemorrhagic disease virus (RHDV): a review. Vet Res. 43(1):12. doi:10.1186/1297-9716-43-12. http://www.veterinaryresearch.org/content/43/1/12.

Abrantes J, Lopes AM, Dalton KP, Melo P, Correia JJ, Ramada M, Alves PC, Parra F, Esteves PJ. 2013. New variant of rabbit hemorrhagic disease virus, Portugal, 2012-2013. Emerg Infect Dis. 19(11):1900–1902. doi:10.3201/eid1911.130908.

Aguayo-adán JA, Santoro S, Rouco C, Delibes-mateos M. 2021. Lack of evidence for differences in the spread of classic (Lagovirus europaeus / GI . 1) and novel (Lagovirus europaeus / GI . 2) rabbit haemorrhagic disease viruses in Europe and North Africa. (April):1–8. doi:10.1002/vetr.1067.

Ahmad S, Elsamadony H, Mahgoub K. 2011. Immunological and Virological Studies on Rabbit Hemorrhagic Disease Virus. Glob Vet. 7.

Alda F, Doadrio I. 2014. Spatial genetic structure across a hybrid zone between European rabbit subspecies. :1–24. doi:10.7717/peerj.582.

Alonso C, Oviedo JM, Mart JM. 1998. Programmed cell death in the pathogenesis of rabbit hemorrhagic disease. :321–332.

Alves JM, Carneiro M, Cheng JY, de Matos AL, Rahman MM, Loog L, Campos PF, Wales N, Eriksson A, Manica A, et al. 2019. Parallel adaptation of rabbit populations to myxoma virus. Science (80-). 363(6433):1319–1326. doi:10.1126/science.aau7285. https://www.science.org/doi/abs/10.1126/science.aau7285.

Ambagala A, Ababio P, Lamboo L, Goolia M, Lung O, Berhane Y. 2021. Outbreak of Rabbit Hemorrhagic Disease Virus 2 Infections, Ghana. (August). doi:10.3201/eid2707.210005.

Anderson RM, May RM. 1982. Coevolution of hosts and parasites. Parasitology. 85(2):411–426.

Andueza A, Lambarri M, Urda V, Prieto I, Villanueva LF, Sánchez-Garcia C. 2018. Evaluacíon del Impacto Económico y Social de la caza en Espana. (April):5–24. https://www.fundacionartemisan.com/wp-content/uploads/2018/05/ESTUDIO-COMPLETO-ESPAÑA.pdf.

Aragão HB. 1927. Myxoma of rabbits. Mem Inst Oswaldo Cruz. 20:225–247.

Argüello-Villares JL. 1991. Viral haemorrhagic disease of rabbits: vaccination and immune response. Rev Sci Tech 10.:459–480.

Arguello-Villares JL, Llanos Pellitero A, Perez Ordoyo Garcia LM. 1988. Enfermedad vírica hemorrágica del conejo en Espana. Med Veterinária. 5:645–650.

Argüello J, Llanos A, Pérez L. 1988. Enfermedad hemorrágica del conejo en España. Med Vet. 5(in Spanish):645–650.

Armour CJ, Thompson V. 1954. SPREAD OF MYXOMATOSIS IN THE FIRST

OUTBREAK I N GREAT BRITAIN. In: Proceedings of the Association of Applied Biologists Ann. Vol. 43. p. 511–518.

Armstrong JA, Pereira HG, Andrewes CH. 1961. Observations on the virus of infectious bovine rhinotracheitis, and its affinity with the Herpesvirus group. Virology. 14:276–285. doi:10.1016/0042-6822(61)90204-5.

Arthur CP, Louzis C. 1988. A review of myxomatosis among rabbits in France. Rev.sci.techOff.int Epiz. 7(4):959–976.

Asin J, Rejmanek D, Clifford DL, Mikolon AB, Henderson EE, Nyaoke AC, Macías-Rioseco M, Streitenberger N, Beingesser J, Woods LW, et al. 2021. Early circulation of rabbit haemorrhagic disease virus type 2 in domestic and wild lagomorphs in southern California, USA (2020-2021). Transbound Emerg Dis. doi:10.1111/tbed.14315.

Bachmann MF, Jennings GT. 2010. Vaccine delivery : a matter of size , geometry , kinetics and molecular patterns. Nat Publ Gr.(October). doi:10.1038/nri2868.

Baily JL, Dagleish MP, Graham M, Maley M, Rocchi MS. 2014. RHDV variant 2 presence detected in Scotland. Vet Rec. 174(16):411.1-411. doi:10.1136/vr.g2781. http://veterinaryrecord.bmj.com/lookup/doi/10.1136/vr.g2781.

Barbieri I, Lavazza A, Brocchi E, Konig M, Capucci L. 1997. Morphological, structural and antigenic modifications of rabbit haemorrhagic disease virus in the course of the disease. :182–193.

Bárcena J, Guerra B, Angulo I, González J, Valcárcel F, Mata CP, Castón JR, Blanco E, Alejo A. 2015. Comparative analysis of rabbit hemorrhagic disease virus (RHDV) and new RHDV2 virus antigenicity, using specific virus-like particles. Vet Res. 46(1):1–6. doi:10.1186/s13567-015-0245-5. http://dx.doi.org/10.1186/s13567-015-0245-5.

Bárcena J, Verdaguer N, Roca R, Morales M, Angulo I, Risco C, Carrascosa JL, Torres JM, Castón JR. 2004. The coat protein of Rabbit hemorrhagic disease virus contains a molecular switch at the N-terminal region facing the inner surface of the capsid. Virology. 322(1):118–134. doi:10.1016/j.virol.2004.01.021.

Barlow A, Lawrence K, Everest D, Dastjerdi A, Finnegan C, Steinbach F. 2014. Confirmation of myxomatosis in a European brown hare in Great Britain. Vet Rec. 175(3):75–76. doi:10.1136/vr.g4621.

Barrett JW, Sypula J, Wang F, Alston LR, Shao Z, Gao X, Irvine TS, McFadden G. 2007. M135R is a novel cell surface virulence factor of myxoma virus. J Virol. 81(1):106–114.

Bartrip PWJ. 2008. Myxomatosis in 1950s Britain. 19(1):83–105. doi:10.1093/tcbh/hwm016.

Belsham GJ, Polacek C, Breum SØ, Larsen LE, Bøtner A. 2010. Detection of myxoma viruses encoding a defective M135R gene from clinical cases of myxomatosis ; possible implications for the role of the M135R protein as a virulence factor. :1–10.

Bertagnoli S, Gelfi J, Petit F, Vautherot JF, Rasschaert D, Laurent S, Le Gall G, Boilletot E, Chantal J, Boucraut-Baralon C. 1996. Protection of rabbits against rabbit viral haemorrhagic disease with a vaccinia-RHDV recombinant virus. Vaccine. 14(6):506–510. doi:10.1016/0264-410x(95)00232-p.

Best SM, Collins S V., Kerr PJ. 2000. Coevolution of host and virus: Cellular localization

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of virus in myxoma virus infection of resistant and susceptible European rabbits. Virology. 277(1):76–91. doi:10.1006/viro.2000.0505.

Best SM, Kerr PJ. 2000. Coevolution of host and virus: the pathogenesis of virulent and attenuated strains of myxoma virus in resistant and susceptible European rabbits. Virology. 267(1):36–48. doi:10.1006/viro.1999.0104.

Blanié S, Mortier J, Delverdier M, Bertagnoli S, Camus-Bouclainville C. 2009. M148R and M149R are two virulence factors for myxoma virus pathogenesis in the European rabbit. Vet Res. 40(1):1.

Boga JA, Casais R, Marin MS, Martin-Alonso JM, Carmenes RS, Prieto M, Parra F. 1994. Molecular cloning, sequencing and expression in Escherichia coli of the capsid protein gene from rabbit haemorrhagic disease virus (Spanish isolate AST/89). J Gen Virol. 75 (Pt 9):2409–2413. doi:10.1099/0022-1317-75-9-2409.

Boga JA, Marín MS, Casais R, Prieto M, Parra F. 1992. In vitro translation of a subgenomic mRNA from purified virions of the Spanish field isolate AST/89 of Rabbit Hemorrhagic Disease Virus (RHDV). Virus Res. 26(1):33–40. doi:10.1016/0168-1702(92)90144-X.

Boga JA, Martín Alonso JM, Casais R, Parra F. 1997. A single dose immunization with rabbit haemorrhagic disease virus major capsid protein produced in Saccharomyces cerevisiae induces protection. J Gen Virol. 78 (Pt 9):2315–2318. doi:10.1099/0022-1317-78-9-2315.

Booy FP, Trus BL, Davison AJ, Steven AC. 1996. The capsid architecture of channel catfish virus, an evolutionarily distant herpesvirus, is largely conserved in the absence of discernible sequence homology with herpes simplex virus. Virology. 215(2):134–141. doi:10.1006/viro.1996.0016.

Bousarghin L, Hubert P, Franzen E, Jacobs N, Boniver J, Delvenne P. 2005. Human papillomavirus 16 virus-like particles use heparan sulfates to bind dendritic cells and colocalize with langerin in Langerhans cells Printed in Great Britain. :1297–1305. doi:10.1099/vir.0.80559-0.

Boyle M, Huntington HP, Boyle M, Flowers GE, Weatherly JW, Hamilton LC, Hinzman L, Gerlach C, Zulueta R, Nicolson C, et al. 2007. The Influence of Human Activity in the Arctic on Climate and Climate Impacts The influence of human activity in the Arctic on climate and climate impacts. (March). doi:10.1007/s10584-006-9162-y.

Branco M, Ferrand N, Monnerot M. 2000. Phylogeography of the European rabbit (Oryctolagus cuniculus) in the Iberian Peninsula inferred from RFLP analysis of the cytochrome b gene. Heredity (Edinb). 85(4):307–317. doi:10.1046/j.1365-2540.2000.00756.x.

Brash ML, Nagy É, Pei Y, Carman S, Emery S, Smith AE, Turner P V. 2010. Acute hemorrhagic and necrotizing pneumonia, splenitis, and dermatitis in a pet rabbit caused by a novel herpesvirus (leporid herpesvirus-4). Can Vet J. 51(12):1383–1386.

Brun A, Saurat P, Gilbert Y, Godart A, Bouquet JF. 1981. Données actuelles sur l'épidémiologie, la pathogénie et la symptomatologie de la myxomatose. Rev Med Vet (Toulouse). 132:585–590.

Bull LB, Dickinson CG. 1937. The specificity of the Virus of Rabbit Myxomatosis. CSIR

J. 10(4):291–294.

Bull LB, Mules MW. 1944. An Investigation of Myxomatosis cuniculi with special Reference to the possible Use of the Disease to control Rabbit Populations in Australia. J Counc Sci Ind Res Aust. 17(2).

Burnet FM. 1952. Myxomatosis as a method of biological control against the Australian rabbit. Am J Public Health Nations Health. 42(12):1522–1526. doi:10.2105/ajph.42.12.1522.

Burnet M. 1968. Changing Patterns.

Cagnin M, Moreno S, Aloise G, Garofalo G, Villafuerte R, Gaona P, Cristaldi M. 1998. Comparative study of Spanish and Italian terrestrial small mammal coenoses from different biotopes in Mediterranean peninsular tip regions. J Biogeogr. 25:1105–1113.

Calaby JH, Gooding CD, Tomlinson AR. 1960. Myxomatosis in Western Australia. CSIRO Wildl Res. 5(2):89–101. https://doi.org/10.1071/CWR9600089.

Calvete C, Capucci L, Lavazza A, Sarto MP, Calvo AJ, Monroy F, Calvo JH. 2021. Changes in European wild rabbit population dynamics and the epidemiology of rabbit haemorrhagic disease in response to artificially increased viral transmission. Transbound Emerg Dis.:1–15. doi:https://doi.org/10.1111/tbed.14421. https://onlinelibrary.wiley.com/doi/abs/10.1111/tbed.14421.

Calvete C, Mendoza M, Sarto M., Jiménez de Bagués MP, Luján L, Molín J, Calvo AJ, Monroy F, Calvo JH. 2019. Detection of Rabbit Hemorrhagic Disease Virus GI . 2 / RHDV2 / B in the Mediterranean Pine Vole (Microtus duodecimcostatus) and White-Toothed Shrew (Crocidura russula). 55(February 2015):1–6. doi:10.7589/2018-05-124.

Calvete C, Mendoza M, Sarto MP, Bagüés MPJ de, Luján L, Molín J, Calvo AJ, Monroy F, Calvo JH. 2019. Detection of Rabbit Hemorrhagic Disease Virus GI.2/RHDV2/b in the Mediterranean Pine Vole (Microtus duodecimcostatus) and White-Toothed Shrew (Crocidura russula). J Wildl Dis. 55(2):467–472. doi:10.7589/2018-05-124.

Calvete C, Sarto P, Calvo AJ, Monroy F, Calvo JH. 2014. Could the new rabbit haemorrhagic disease virus variant (RHDVb) be fully replacing classical RHD strains in the Iberian Peninsula ? World Rabbit Sci. 22:91–91. doi:10.4995/wrs.2014.1715.

Camarda A, Pugliese N, Cavadini P, Circella E, Capucci L, Caroli A, Legretto M, Mallia E, Lavazza A. 2014. Detection of the new emerging rabbit haemorrhagic disease type 2 virus (RHDV2) in Sicily from rabbit (Oryctolagus cuniculus) and Italian hare (Lepus corsicanus). Res Vet Sci. 97(3):642–645. doi:10.1016/j.rvsc.2014.10.008. http://dx.doi.org/10.1016/j.rvsc.2014.10.008.

Cameron C, Hota-Mitchell S, Chen L, Barrett J, Cao JX, Macaulay C, Willer D, Evans D, McFadden G. 1999. The complete dna sequence of myxoma virus. Virology. 264(2):298–318. doi:10.1006/viro.1999.0001.

Camus-Bouclainville C, Gretillat M, Py R, Gelfi J, Guérin JL, Bertagnoli S. 2011. Genome sequence of SG33 strain and recombination between wild-type and vaccine myxoma viruses. Emerg Infect Dis. 17(4):633–638. doi:10.3201/eid1704.101146.

Capucci L, Cavadini P, Schiavitto M, Lombardi G, Lavazza A. 2017. Increased pathogenicity in rabbit haemorrhagic disease virus type 2 (RHDV2). Vet Rec. 180(17):426.2-426. doi:10.1136/vr.104132.

http://veterinaryrecord.bmj.com/lookup/doi/10.1136/vr.104132.

Capucci L, Fallacara F, Grazioli S, Lavazza A, Pacciarini ML, Brocchi E. 1998. A further step in the evolution of rabbit hemorrhagic disease virus: The appearance of the first consistent antigenic variant. Virus Res. 58(1–2):115–126. doi:10.1016/S0168-1702(98)00106-3.

Capucci L, Frigoli G, Ronshold L, Lavazza A, Brocchi E, Rossi C. 1995. Antigenicity of the rabbit hemorrhagic disease virus studied by its reactivity with monoclonal antibodies Lorenzo. Virus Res. 37:221–238.

Capucci L, Fusi P, Lavazza a, Pacciarini ML, Rossi C. 1996. Detection and preliminary characterization of a new rabbit calicivirus related to rabbit hemorrhagic disease virus but nonpathogenic. J Virol. 70(12):8614–8623.

Capucci L, Scicluna M, Lavazza A. 1991. Diagnosis of viral haemorrhagic disease of rabbits and the European brown hare syndrome. Rev Sci Tech. 10:347–370.

Carvalho, Abade Dos Santos FA, Monteiro M, Carvalho P, Mendonça P, Duarte MD. 2020. First cases of myxomatosis in Iberian hares (Lepus granatensis) in Portugal. Vet Rec Case Reports. 8(2):1–5. doi:10.1136/vetreccr-2019-001044. https://vetrecordcasereports.bmj.com/content/8/2/e001044.

Carvalho C, Abade dos Santos FA, Monteiro J, Duarte M, Alves PC, Hora A, Lopes G. 2020. A importância dos leporídeos no equilíbrio das cadeias tróficas e na biodiversidade ibérica. Vida Rural.(July/August):40–48. doi:10.13140/RG.2.2.34393.54889.

Carvalho CL, Abade dos Santos F.A., Fagulha T, Carvalho P, Mendonça P, Monteiro M, Duarte MD. 2020. Myxoma virus and rabbit haemorrhagic disease virus 2 coinfection in a European wild rabbit (Oryctolagus cuniculus algirus), Portugal. :18–21. doi:10.1136/vetreccr-2019-001002.

Carvalho CL, Abade dos Santos Fábio A., Fagulha T, Carvalho P, Mendonça P, Monteiro M, Duarte MD. 2020. Myxoma virus and rabbit haemorrhagic disease virus 2 coinfection in a European wild rabbit (Oryctolagus cuniculus algirus), Portugal. :18–21. doi:10.1136/vetreccr-2019-001002.

Carvalho CL, Leclerc Duarte E, Monteiro JM, Afonso C, Pacheco J, Carvalho P, Mendonça P, Botelho A, Albuquerque T, Themudo P, et al. 2017. Progression of rabbit haemorrhagic disease virus 2 upon vaccination in an industrial rabbitry: A laboratorial approach. World Rabbit Sci. 25(1):73–85. doi:10.4995/wrs.2017.5708.

Carvalho, Silva S, Gouveia P, Costa M, Duarte EL, Henriques AM, Barros SS, Luís T, Ramos F, Fagulha T, et al. 2017. Emergence of rabbit haemorrhagic disease virus 2 in the archipelago of Madeira, Portugal (2016–2017). Virus Genes.:1–5. doi:10.1007/s11262-017-1483-6.

Cavadini P, Botti G, Barbieri I, Lavazza A, Capucci L. 2010. Molecular characterization of SG33 and Borghi vaccines used against myxomatosis. Vaccine. 28(33):5414–5420. doi:10.1016/j.vaccine.2010.06.017. http://dx.doi.org/10.1016/j.vaccine.2010.06.017.

CDFW CD of F and W. 2021. Mountain lions in California. https://wildlife.ca.gov/Conservation/Mammals/ Mountain-Lion.

Cesarman E. 2011. Gammaherpesvirus and lymphoproliferative disorders in

immunocompromised patients. Cancer Lett. 305(2):163–174. doi:10.1016/j.canlet.2011.03.003.

Chasey D. 1997. Rabbit haemorrhagic disease : the new scourge of Oryctolagus cuniculus. Lab Anim.(31):33–44.

Chasey D, Lucas M, Westcott D, Williams M. 1992. European brown hare syndrome in the U.K.; a calicivirus related to but distinct from that of viral haemorrhagic disease in rabbits. Arch Virol. 124:363-370.

Chen L, Liu G, Ni Z, Yu B, Yun T, Song Y, Hua J, Li S, Chen J. 2009. Minor structural protein VP2 in rabbit hemorrhagic disease virus downregulates the expression of the viral capsid protein VP60. J Gen Virol. 90(12):2952–2955. doi:10.1099/vir.0.015081-0.

CNDDB CNDD. 2021. Special animals list. https://nrm.dfg.ca.gov/FileHandler.ashx?DocumentID=109406&inline.

Collins JJ. 1955. Myxomatosis in the common hare. Ir Vet J. 9, 268.

Cooke BD. 1983. Changes in the age-structure and size of populations of wild rabbits in South Australia, following the introduction of European rabbit fleas, Spilopsyllus cuniculi (Dale), as vectors of myxomatosis. Wildl Res. 10(1):105–120.

Cooke BD, Robinson AJ, Merchant JC, Nardin A, Capucci ML. 2000. Use of ELISAs in field studies of rabbit haemorrhagic disease (RHD) in Australia. Epidemiol Infect. 124(October 1995):563–576. doi:10.1017/S0950268899003994.

Cowling RM, Rundel PW, Lamont BB, Arroyo MK, Arianoutsou M. 1996. Plant diversity in mediterranean-climate regions. TREE. 11(9). doi:https://doi.org/10.1016/0169-5347(96)10044-6.

Dalton K, Nicieza I, Podadera A, Llano D, Martin Alonso J, Ocaña M, Vázquez F, Velasco B, Landeta O, Parra F. 2017. Fast specific field detection of RHDVb. Transbound Emerg Dis. 65. doi:10.1111/tbed.12607.

Dalton KP, Abrantes J, Lopes AM, Nicieza I, Álvarez ÁL, Esteves PJ, Parra F. 2015. Complete genome sequence of two rabbit hemorrhagic disease virus variant b isolates detected on the Iberian Peninsula. Arch Virol. 160:877–881. doi:10.1007/s00705-014-2329-3.

Dalton KP, Alvarado C, Reytor E, Del Carmen Nuñez M, Podadera A, Martínez-Alonso D, Alonso JMM, Nicieza I, Gómez-Sebastián S, Dalton RM, et al. 2021. Chimeric VLPs Bearing VP60 from Two Serotypes of Rabbit Haemorrhagic Disease Virus Are Protective against Both Viruses. Vaccines. 9(9). doi:10.3390/vaccines9091005.

Dalton KP, Arnal JL, Benito AA, Chacón G, Martín Alonso JM, Parra F. 2018. Conventional and real time RT-PCR assays for the detection and differentiation of variant rabbit hemorrhagic disease virus (RHDVb) and its recombinants. J Virol Methods. 251(October 2017):118–122. doi:10.1016/j.jviromet.2017.10.009. http://dx.doi.org/10.1016/j.jviromet.2017.10.009.

Dalton KP, Martín JM, Nicieza I, Podadera A, Llano D, Casais R, Gimenez S, Badiola I, Agüero M, Duran M, et al. 2019. Myxoma virus jumps species to the Iberian hare. Transbound Emerg Dis.:1–9. doi:10.1111/tbed.13296. https://onlinelibrary.wiley.com/doi/abs/10.1111/tbed.13296.

Dalton KP, Nicieza I, Abrantes J, Esteves PJ, Parra F. 2014. Spread of new variant RHDV in domestic rabbits on the Iberian Peninsula. Vet Microbiol. 169(1–2):67–73. doi:10.1016/j.vetmic.2013.12.015.

Dalton KP, Nicieza I, Balseiro A, Muguerza MA, Rosell JM, Casais R, Álvarez ÁL, Parra F. 2012. Variant rabbit hemorrhagic disease virus in young rabbits, Spain. Emerg Infect Dis. 18(12):2009–2012. doi:10.3201/eid1812.120341.

Dalton KP, Nicieza I, Baragão A, Alonso JMM, Parra F. 2010. Molecular characterisation of virulence graded field isolates of myxoma virus. Virol J. 7:1–12. doi:10.1186/1743-422X-7-49.

Daughenbaugh KF, Fraser CS, Hershey JWB, Hardy ME. 2003. The genome-linked protein VPg of the Norwalk virus binds eIF3, suggesting its role in translation initiation complex recruitment. EMBO J. 22(11):2852–2859. doi:10.1093/emboj/cdg251.

Davison AJ, Richard E, Bernhard E, Gary HS, Duncan MJ, Anthony MC, Philip PE, Bernard R, Michael SJ, Etienne T. 2009. The order of Herpesvirales. Arch Virol. 154(1):171–177. doi:10.1007/s00705-008-0278-4.

Davison AJ, Trus BL, Cheng N, Steven AC, Watson MS, Cunningham C, Deuff R-M Le, Renault T. 2005. A novel class of herpesvirus with bivalve hosts. J Gen Virol. 86(Pt 1):41–53. doi:10.1099/vir.0.80382-0.

Delibes-Mateos M, Delibes M, Ferreras P, Villafuerte R. 2008. Key role of European rabbits in the conservation of the western Mediterranean Basin hotspot. Conserv Biol. 22(5):1106–1117. doi:10.1111/j.1523-1739.2008.00993.x.

Delibes-Mateos M, Ferreira C, Rouco C, Villafuerte R, Barrio IC. 2014. Conservationists, hunters and farmers: the European rabbit Oryctolagus cuniculus management conflict in the Iberian Peninsula. Mamm Rev. 44(3–4):190–203. doi:https://doi.org/10.1111/mam.12022. https://onlinelibrary.wiley.com/doi/abs/10.1111/mam.12022.

Delibes-Mateos M, Ferreras P, Villafuerte R. 2009. European rabbit population trends and associated factors: A review of the situation in the Iberian Peninsula. Mamm Rev. 39(2):124–140. doi:10.1111/j.1365-2907.2009.00140.x.

Delibes-Mateos M, Redpath SM, Angulo E, Ferreras P, Villafuerte R. 2007. Rabbits as a keystone species in southern Europe. Biol Conserv. 137(1):149–156. doi:10.1016/j.biocon.2007.01.024.

Delibes M, Hiraldo F. 1981. The rabbit as a prey in the Iberian mediterranean ecossystem. In: Myers K, C.D. M, editors. Proceedings of the World Lagomorph Conference (1979). University of Guelph, Ontario. p. 654–663.

Dellafiore CM, Rouco C, Muñóz Vallés S, Gallego Fernández JB. 2014. Seasonal habitat use by the European rabbit (Oryctolagus cuniculus) in a coastal dune system in SW Spain. Anim Biodivers Conserv. 37.2:233–242.

Devanter DRVAN, Warrener P, Bennett L, Schultz ER, Coulter S, Garber RL, Rose TM, Black J. 1996. Detection and Analysis of Diverse Herpesviral Species by Consensus Primer PCR. 34(7):1666–1671.

Duarte M, Carvalho C, Bernardo S, Barros S, Benevides S, Flor L, Monteiro M, Marques I, Henriques M, Barros SC, et al. 2015. Rabbit haemorrhagic disease virus 2 (RHDV2)

outbreak in Azores: Disclosure of common genetic markers and phylogenetic segregation within the European strains. Infect Genet Evol. 35:163–171. doi:10.1016/j.meegid.2015.08.005. http://dx.doi.org/10.1016/j.meegid.2015.08.005.

Duarte M.D., Barros SC, Henriques AM, Fagulha MT, Ramos F, Luís T, Fevereiro M. 2014. Development and validation of a real time PCR for the detection of myxoma virus based on the diploid gene M000 . 5L / R. J Virol Methods. 196:219–224. doi:10.1016/j.jviromet.2013.11.014. http://dx.doi.org/10.1016/j.jviromet.2013.11.014.

Duarte MD, Carvalho C, Abade dos Santos FA, Monteiro J, Monteiro M, Carvalho P, Mendonça P, Tavares Santos P, Melo P. 2020. The Health and Future of the Six Hare Species in Europe: A Closer Look at the Iberian Hare. In: Lagomorphs. IntechOpen (in press).

Duarte MD, Carvalho CL, Abade dos Santos FA, Gomes J, Alves PC, Esteves PJ, Abrantes J, Lopes AM, P. M, Serronha A, et al. 2018. +Coelho: Avaliação Ecossanitária das Populações Naturais de Coelho Bravo Visando o Controlo da Doença Hemorrágica Viral.

Duarte MD, Carvalho CL, Abade dos Santos FA, Gomes J, Alves PC, Esteves PJ, Abrantes J, Lopes AM, P. M, Serronha A, et al. 2021. +Coelho 2: Desenvolvimento e implementação de medidas práticas impulsionadoras da recuperação dos leporídeos silvestres em Portugal. Lisbon: +Coelho 2 GT.

Duarte MD, Carvalho CL, Barros SC, Henriques AM, Ramos F, Fagulha T, Luís T, Duarte EL, Fevereiro M. 2015. A real time Taqman RT-PCR for the detection of rabbit hemorrhagic disease virus 2 (RHDV2). J Virol Methods. 219:90–95. doi:10.1016/j.jviromet.2015.03.017. http://dx.doi.org/10.1016/j.jviromet.2015.03.017.

Duarte Margarida Dias, Henriques AM, Barros S, Luís T, Fagulha T, Ramos F, Fevereiro M. 2014. 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. Infect Genet Evol. 27:149–155. doi:10.1016/j.meegid.2014.07.017. http://dx.doi.org/10.1016/j.meegid.2014.07.017.

Duclos P, Tuaillon P, Joubert L. 1983. Histopathologie de l ' atteinte cutanéo-muqueuse et pulmonaire de la myxomatose. Bull Acad Vet. 56 (in Fre:95–104.

Dwyer G, Levin SA, Buttel L. 1990. A simulation model of the population dynamics and evolution of myxomatosis. Ecol Monogr. 60(4):423–447.

Elsworth P, Cooke BD, Kovaliski J, Sinclair R, Holmes EC, Strive T. 2014. Increased virulence of rabbit haemorrhagic disease virus associated with genetic resistance in wild Australian rabbits (Oryctolagus cuniculus). Virology. 464–465(1):415–423. doi:10.1016/j.virol.2014.06.037. http://dx.doi.org/10.1016/j.virol.2014.06.037.

Elsworth PG, Kovaliski J, D. CB. 2012. Rabbit haemorrhagic disease: are Australian rabbits (Oryctolagus cuniculus) evolving resistance to infection with Czech CAPM 351 RHDV? Epidemiol Infect. 140(11):1972–1981. doi:10.1017/S0950268811002743. http://www.journals.cambridge.org/abstract_S0950268811002743.

Erlach RFG, Ildfell RJB. 2008. An Outbreak of Fatal Herpesvirus Infection in Domestic Rabbits in Alaska. 374:369–374.

Esteves PJ, Abrantes J, Bertagnoli S, Cavadini P. 2015. Emergence of Pathogenicity in Lagoviruses : Evolution from Pre-existing Nonpathogenic Strains or through a Species Jump ? :56–61. doi:10.1371/journal.ppat.1005087.

Falke D, Siegert R, Vogell W. 1959. Electron microscopic findings on the problem of double membrane formation in herpes simplex virus. Arch Gesamte Virusforsch. 9:484–496.

Family BG, Ruvoën-clouet N, Ganière JP, André-fontaine G, Blanchard D, Pendu JLE. 2001. Binding of Rabbit Hemorrhagic Disease Virus to Antigens of the ABH Histo-Binding of Rabbit Hemorrhagic Disease Virus to Antigens of the ABH Histo-Blood Group Family. (January). doi:10.1128/JVI.74.24.11950-11954.2000.

Fenner BYF, Marshall ID. 1957. A comparison of the virulence for european rabbits (Oryctolagus cuniculus) of strains of Myxoma virus recovered in the field in Australia , Europe and America. J Hyg (Lond). 55 (2):149–191.

Fenner F. 2010. Deliberate introduction of the European rabbit, Oryctolagus cuniculus, into Australia. Rev Sci Tech. 29:103–111.

Fenner F, Day MF, Woodroofe GM. 1956. Epidemiological consequences of the mechanical transmission of myxomatosis by mosquitoes. Epidemiol Infect. 54(2):284–303.

Fenner F., Fantini B. 1999. History of Myxomatosis - an Experiment in Evolution. In: Biological Control of Vertebrate Pests. CABI. New York, USA.

Fenner F, Fantini B. 1999. Biological Control of vertebrate pests: the history of myxomatosis - an experiment in evolution.

Fenner F, Ratcliffe FN. 1965. Myxomatosis. Cambridge Univ Press London, UK.

Fenner F, Woodroof GM. 1954. PROTECTION OF LABORATORY RABBITS AGAINST MYXO-MATOSIS BY VACCINATION WITH FIBROMA VIRUS. Aust J Exp Biol Med Sci. 32(5).

Fenner F, Woodroofe GM. 1953. The pathogenesis of infectious myxomatosis; the mechanism of infection and the immunological response in the European rabbit (Oryctolagus cuniculus). Br J Exp Pathol. 34(4):400–411.

Fenner F, Woodroofe GM. 1965. Changes in the virulence and antigenic structure of strains of myxoma virus recovered from Australian wild rabbits between 1950 and 1964. Aust J Exp Biol Med Sci. 43:359–370.

Fenner FJ. 1983. The Florey Lecture, 1983-Biological control, as exemplified by smallpox eradication and myxomatosis. Proc R Soc London Ser B Biol Sci. 218(1212):259–285.

Fernández E, Toledo JR, Chiong M, Parra F, Rodríguez E, Montero C, Méndez L, Capucci L, Farnós O. 2011. Veterinary Immunology and Immunopathology Single dose adenovirus vectored vaccine induces a potent and long-lasting immune response against rabbit hemorrhagic disease virus after parenteral or mucosal administration. 142:179–188. doi:10.1016/j.vetimm.2011.05.007.

Fernández E, Toledo JR, Gonza N, Marti M, Limonta M, Sa K, Rodri A. 2013. Conformational and Thermal Stability Improvements for the Large-Scale Production of

Yeast-Derived Rabbit Hemorrhagic Disease Virus-Like Particles as Multipurpose Vaccine. PLoS One. 8(2). doi:10.1371/journal.pone.0056417.

Ferrand N. 1995. Variação genética de proteinas em populações de coelho (Oryctolagus cuniculus) Analise da diferenciação subespecifica, subestruturação, expansão sgeografica e domesticação. University of Porto (Portugal).

Ferreira A, Ferreira AJ. 2014. Post-weaning growth of endemic iberian wild rabbit subspecies, oryctolagus cuniculus algirus, KEPT in a semi-extensive enclosure: Implications for management and conservation. World Rabbit Sci. 22(2):129–136. doi:10.4995/wrs.2014.1673.

Ferreira C. 2012. European rabbit research in the Iberian Peninsula: State of the art and future perspectives. Eur J Wildl Res. 58(6):885–895. doi:10.1007/s10344-012-0664-9.

Ferreira C, Delibes-Mateos M. 2010. Wild Rabbit Management in the Iberian Peninsula: State of the Art and Future Perspectives for Iberian Lynx Conservation. Wildl Biol Pract. 6(3):48–66. doi:10.2461/wbp.lynx.4. http://socpvs.org/journals/index.php/wbp/article/view/10.2461-wbp.lynx.4.

Ferreira C, Ramírez E, Castro F, Ferreras P, Alves PC, Redpath S, Villafuerte R. 2009. Field experimental vaccination campaigns against myxomatosis and their effectiveness in the wild. Vaccine. 27(50):6998–7002. doi:10.1016/j.vaccine.2009.09.075.

Flux JEC, Fullagar PJ. 1992. World distribution of the Rabbit Oryctolagus funiculus on islands. Mamm Rev. 22(3–4):151–205. doi:https://doi.org/10.1111/j.1365-2907.1992.tb00129.x. https://onlinelibrary.wiley.com/doi/abs/10.1111/j.1365-2907.1992.tb00129.x.

Forrester NL, Boag B, Buckley A, Moureau G, Gould EA. 2009. Co-circulation of widely disparate strains of Rabbit haemorrhagic disease virus could explain localised epidemicity in the United Kingdom. Virology. 393(1):42–48. doi:10.1016/j.virol.2009.07.008. http://dx.doi.org/10.1016/j.virol.2009.07.008.

Forrester NL, Trout RC, Gould EA. 2007. Benign circulation of rabbit haemorrhagic disease virus on Lambay Island , Eire ☆. 358:18–22. doi:10.1016/j.virol.2006.09.011.

Fresco-Taboada A, Montón M, Tapia I, Soria E, Bárcena J, Guillou-Cloarec C, Le Gall-Reculé G, Blanco E, Rueda P. 2022. Development and Evaluation of a Duplex Lateral Flow Assay for the Detection and Differentiation between Rabbit Haemorrhagic Disease Virus Lagovirus europaeus/GI.1 and /GI.2. Biology (Basel). 11(3). doi:10.3390/biology11030401. https://www.mdpi.com/2079-7737/11/3/401.

Fukui H, Shimoda H, Kadekaru S, Henmi C. 2021. Rabbit hemorrhagic disease virus type 2 epidemic in a rabbit colony in Japan. :841–845. doi:10.1292/jvms.21-0007.

Furlong D, Swift H, Roizman B. 1972. Arrangement of herpesvirus deoxyribonucleic acid in the core. J Virol. 10(5):1071–1074. doi:10.1128/JVI.10.5.1071-1074.1972.

Furukawa S, Kuroda Y, Sugiyama A. 2014. A Comparison of the Histological Structure of the Placenta in Experimental Animals. :11–18. doi:10.1293/tox.2013-0060.

Le Gall-Reculé G, Lavazza A, Marchandeau S, Bertagnoli S, Zwingelstein F, Cavadini P, Martinelli N, Lombardi G, Guérin JL, Lemaitre E, et al. 2013. Emergence of a new lagovirus related to rabbit haemorrhagic disease virus. Vet Res. 44(1):1–13.

doi:10.1186/1297-9716-44-81.

Le Gall-Recule G, Zwingelstein F, Boucher S, Le Normand B, Plassiart G, Portejoie Y, Decors A, Bertagnoli S, Guerin J-L, Marchandeau S. 2011. Detection of a new variant of rabbit haemorrhagic disease virus in France. Vet Rec. 168(5):137–138. doi:10.1136/vr.d697. http://veterinaryrecord.bmj.com/cgi/doi/10.1136/vr.d697.

Gall A, Hoffmann B, Teifke JP, Lange B, Schirrmeier H. 2007. Persistence of viral RNA in rabbits which overcome an experimental RHDV infection detected by a highly sensitive multiplex real-time RT-PCR. Vet Microbiol. 120(1–2):17–32. doi:10.1016/j.vetmic.2006.10.006.

Le Gall G, Arnauld C, Boilletot E, Morisse JP, Rasschaert D. 1998. Molecular epidemiology of rabbit haemorrhagic disease virus outbreaks in France during 1988 to 1995. J Gen Virol. 79(1):11–16. doi:10.1099/0022-1317-79-1-11.

García-Bocanegra I, Camacho-Sillero L, Caballero-Gómez J, Aguero M, Gómez-Guillamón F, Ruiz-Casas J, Díaz-Cao J, García E, Ruano M, Haza R. 2020. Monitoring of emerging myxoma virus epidemics in Iberian hares (. Transbound Emerg Dis. 68(3):1275–1282. doi:10.1111/tbed.13781.

García-Bocanegra I, Camacho-Sillero L, Risalde MA, Dalton K, Caballero-Gómez J, Aguero M, Zorrila I, Gómez-Guillamón F. 2019. First outbreak of myxomatosis in Iberian hares (Lepus granatensis). Transbound Emerg Dis.(June):1–5. doi:10.1111/tbed.13289.

García-Lastra R, San-Miguel B, Crespo I, Jorquera F, Alvarez M, González-Gallego J, Tunon MJ. 2010. Signaling pathways involved in liver injury and regeneration in rabbit hemorrhagic disease, an animal model of virally-induced fulminant hepatic failure. Vet Res. 41(1). doi:10.1051/vetres/2009050.

Gehrmann B (Bezirksinstitut fuer VS (Germany)), Kretzschmar C. 1991. Experimental investigations on Rabbit Haemorrhagic Disease (RHD) - transmission by flies. Berliner und Muenchener Tieraerztliche Wochenschrift (Germany, FR). v. 104.

Gelfi J, Chantal J, Phong TT, Py R, Boucraut-Baralon C. 1999. Development of an ELISA for detection of myxoma virus-specific rabbit antibodies: test evaluation for diagnostic applications on vaccinated and wild rabbit sera. J Vet diagnostic Investig Off Publ Am Assoc Vet Lab Diagnosticians, Inc. 11(3):240–245. doi:10.1177/104063879901100306.

Gelmetti D, Grieco V, Rossi C, Capucci L, Lavazza A. 1998. Detection of rabbit haemorrhagic disease virus (RHDV) by in situ hybridisation with a digoxigenin labelled RNA probe. J Virol Methods. 72(2):219–226. doi:10.1016/S0166-0934(98)00030-5.

Geraldes A, Ferrand N, Nachman MW. 2006. Contrasting patterns of introgression at X-linked loci across the hybrid zone between subspecies of the European rabbit (Oryctolagus cuniculus). Genetics. 173(2):919–933. doi:10.1534/genetics.105.054106.

Gibb JA. 1990. The European Rabbit. In: Chapman JA, Flux JE, editors. Rabbits, hares and pikas: status survey and conservation action plan. IUCN, Switzerland. p. 116–120.

Gibson W. 1996. Structure and assembly of the virion. Intervirology. 39(5–6):389–400. doi:10.1159/000150509.

Gómez Sal A, Rey Benayas JM, López-Pintor A, Rebollo S. 1999. Role of disturbance in maintaining a savanna-like pattern in Mediterranean Retama sphaerocarpa shrubland.

J Veg Sci. 10:365–370.

Goodfellow I, Chaudhry Y, Gioldasi I, Gerondopoulos A, Natoni A, Labrie L, Laliberté J-F, Roberts L. 2005. Calicivirus translation initiation requires an interaction between VPg and eIF4E. EMBO Rep. 6(10):968–972. doi:10.1038/sj.embor.7400510. http://embor.embopress.org/cgi/doi/10.1038/sj.embor.7400510.

Gould AR, Kattenbelt J a, Lenghaus C, Morrissy C, Chamberlain T, Collins BJ, Westbury H a. 1997. The complete nucleotide sequence of rabbit haemorrhagic disease virus (Czech strain V351): use of the polymerase chain reaction to detect replication in Australian vertebrates and analysis of viral population sequence variation. Virus Res. 47:7–17.

Granzow H, Weiland F, Strebelow HG, Liu CM, Schirrmeier H. 1996. Rabbit hemorrhagic disease virus (RHDV): Ultrastructure and biochemical studies of typical and core-like particles present in liver homogenates. Virus Res. 41(2):163–172. doi:10.1016/0168-1702(96)01285-3.

Gregg D, House C, Meyer R, Berninger M. 1991. Viral haemorrhagic disease of rabbits in Mexico: epidemiology and viral characterization. Rev Sci Tech. 10:435–451.

Grest P, Albicker P, Hoelzle L, Wild P, Pospischil A. 2002. Herpes Simplex Encephalitis in a Domestic Rabbit (Oryctolagus cuniculus). 126:308–311. doi:10.1053/jcpa.2002.0548.

Griffiths P. 1999. Time to consider the concept of a commensal virus? Rev Med Virol. 9(2):73–74. doi:10.1002/(SICI)1099-1654(199904/06)9:2<73::AID-RMV254>3.0.CO;2-5.

Gruber A, Pakozdy A, Weissenbock H, Csokai J, Kunzel F. 2009. A Retrospective Study of Neurological Disease in 118 Rabbits. J Comp Pathol. 140:31–37. doi:10.1016/j.jcpa.2008.09.009.

Guillon P, Ruvoën-clouet N, Le Moullac-Vaidye B, Marchandeau S, Le Pendu J. 2009. Association between expression of the H histo-blood group antigen, α1,2fucosyltransferases polymorphism of wild rabbits, and sensitivity to rabbit hemorrhagic disease virus. Glycobiology. 19(1):21–28. doi:10.1093/glycob/cwn098.

Guitton J-S, Devillard S, Guénézan M, Fouchet D, Pontier D, Marchandeau S. 2008. Vaccination of free-living juvenile wild rabbits (Oryctolagus cuniculus) against myxomatosis improved their survival. Prev Vet Med. 84(1–2):1–10.

Guittre C, Baginski I, Le Gall G, Prave M, Trepo C, Cova L. 1995. Detection of rabbit haemorrhagic disease virus isolates and sequence comparison of the N-terminus of the capsid protein gene by the polymerase chain reaction. Res Vet Sci. 58(2):128–132. doi:10.1016/0034-5288(95)90065-9. http://www.sciencedirect.com/science/article/B6WWR-4CWRXPY-2C/2/f53c8c366ed8a00e8c273e1c73412d1a.

Guo H, Zhu J, Tan Y, Li C, Chen Z, Sun S, Liu G. 2016. Self-assembly of virus-like particles of rabbit hemorrhagic disease virus capsid protein expressed in Escherichia coli and their immunogenicity in rabbits. Antiviral Res. 131:85–91. doi:10.1016/j.antiviral.2016.04.011. http://dx.doi.org/10.1016/j.antiviral.2016.04.011.

Hall RN, Mahar JE, Haboury S, Stevens V, Holmes EC, Strive T. 2015. Emerging rabbit

hemorrhagic disease virus 2 (RHDVb), Australia. Emerg Infect Dis. 21(12):2276–2278. doi:10.3201/eid2112.151210.

Hall RN, Mahar JE, Read AJ, Mourant R, Piper M, Huang N, Strive T. 2018. A strain-specific multiplex RT-PCR for Australian rabbit haemorrhagic disease viruses uncovers a new recombinant virus variant in rabbits and hares. Transbound Emerg Dis. 65(2):e444–e456. doi:https://doi.org/10.1111/tbed.12779. https://onlinelibrary.wiley.com/doi/abs/10.1111/tbed.12779.

Hall RN, Peacock DE, Kovaliski J, Mahar JE, Mourant R, Piper M, Strive T. 2017. Detection of RHDV2 in European brown hares (Lepus europaeus) in Australia. Vet Rec. 180(5):121. doi:10.1136/vr.104034.

Henning J, Meers J, Davies PR, Morris RS. 2005. Survival of rabbit haemorrhagic disease virus (RHDV) in the environment. Epidemiol Infect. 133(4):719–30. doi:10.1017/S0950268805003766. http://www.ncbi.nlm.nih.gov/pubmed/16050519%5Cnhttp://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC2870301.

Hesselton RM, Yang WC, Medveczky P, Sullivan JL. 1988. Pathogenesis of Herpesvirus sylvilagus infection in cottontail rabbits. Am J Pathol. 133(3):639–647.

Hinze HC. 1971. New Member of the Herpesvirus Group Isolated from Wild Cottontail Rabbits. 3(2):350–354.

Hobbs J. 1928. Studies of the nature of infectious myxoma of rabbits. Am J Hyg. 8:800–839.

Hu B, Wei H, Fan Z, Song Y, Chen M, Qiu R, Zhu W, Xu W, Xue J, Wang F. 2021. Emergence of rabbit haemorrhagic disease virus 2 in China in 2020. Vet Med Sci. 7(1):236–239. doi:https://doi.org/10.1002/vms3.332. https://onlinelibrary.wiley.com/doi/abs/10.1002/vms3.332.

Hu Z, Tian X, Zhai Y, Xu W, Zheng D, Sun F. 2010. Cryo-electron microscopy reconstructions of two types of wild rabbit hemorrhagic disease viruses characterized the structural features of Lagovirus. Protein Cell. 1(1):48–58. doi:10.1007/s13238-010-0007-0.

Hurst EW. 1937. Myxoma and the Shope fibroma. I. The histology of myxoma. Br J Exp Pathol. 18:1–15.

ICTV IC on T of V. 2021. Genus: Lagovirus. [accessed 2021 Jan 10]. https://talk.ictvonline.org/ictvreports/ictv_online_report/positive-sense-rna-viruses/w/caliciviridae/1163/genus-lagovirus.

Jin L, Löhr C V., Vanarsdall AL, Baker RJ, Moerdyk-Schauwecker M, Levine C, Gerlach RF, Cohen SA, Alvarado DE, Rohrmann GF. 2008. Characterization of a novel alphaherpesvirus associated with fatal infections of domestic rabbits. Virology. 378(1):13–20. doi:10.1016/j.virol.2008.05.003.

Jin L, Valentine BA, Baker RJ, Löhr C V., Gerlach RF, Bildfell RJ, Moerdyk-Schauwecker M. 2008. An outbreak of fatal herpesvirus infection in domestic rabbits in alaska. Vet Pathol. 45(3):369–374. doi:10.1354/vp.45-3-369.

Johannsen E, Luftig M, Chase MR, Weicksel S, Cahir-McFarland E, Illanes D, Sarracino D, Kieff E. 2004. Proteins of purified Epstein-Barr virus. Proc Natl Acad Sci.

101(46):16286–16291.   doi:10.1073/pnas.0407320101.
https://www.pnas.org/content/101/46/16286.

Johnston SL, Siegel CS. 1990. Comparison of enzyme immunoassay, shell vial culture, and conventional cell culture for the rapid detection of herpes simplex virus. Diagn Microbiol Infect Dis. 13(3):241–244. doi:10.1016/0732-8893(90)90066-5.

Joubert L. 1973. La Myxomatose T.II.

Joubert L, Leftheriotis E. 1972. La Myxomatose. LEPINE P, GORET P, editors. http://ir.obihiro.ac.jp/dspace/handle/10322/3933.

Kattenhorn LM, Mills R, Wagner M, Lomsadze A, Makeev V, Borodovsky M, Ploegh HL, Kessler BM. 2004. Identification of proteins associated with murine cytomegalovirus virions. J Virol. 78(20):11187–11197. doi:10.1128/JVI.78.20.11187-11197.2004.

Kerr P, McFadden G. 2002. Immune responses to myxoma virus. Viral Immunol. 15(2):229–246. doi:10.1089/08828240260066198.

Kerr PJ. 1997. An ELISA for Epidemiological Studies of Myxomatosis: Persistence of Antibodies to Myxoma Virus in European Rabbits (Oryctolagus cuniculus). Wildl Res. 24:53–65.

Kerr PJ. 2012. Myxomatosis in Australia and Europe: A model for emerging infectious diseases. Antiviral Res. 93(3):387–415. doi:10.1016/j.antiviral.2012.01.009. http://dx.doi.org/10.1016/j.antiviral.2012.01.009.

Kerr PJ, Ghedin E, Depasse J V, Fitch A, Cattadori IM, Hudson PJ, Tscharke DC, Read AF, Holmes EC. 2012. Evolutionary History and Attenuation of Myxoma Virus on Two Continents. 8(10). doi:10.1371/journal.ppat.1002950.

Kerr PJ, Hone J, Perrin L, French N, Williams CK. 2010. Molecular and serological analysis of the epidemiology of myxoma virus in rabbits. Vet Microbiol. 143(2–4):167–178.

Kerr PJ, Kitchen A, Holmes EC. 2009. Origin and phylodynamics of rabbit hemorrhagic disease virus. J Virol. 83(23):12129–12138. doi:10.1128/jvi.01523-09. http://www.scopus.com/inward/record.url?eid=2-s2.0-70450191608&partnerID=tZOtx3y1.

Kerr PJ, Liu J, Cattadori I, Ghedin E, Read AF, Holmes EC. 2015. Myxoma virus and the leporipoxviruses: An evolutionary paradigm. Viruses. 7(3):1020–1061. doi:10.3390/v7031020.

Kerr PJ, Rogers MB, Fitch A, DePasse J V., Cattadori IM, Hudson PJ, Tscharke DC, Holmes EC, Ghedin E. 2013. Comparative Analysis of the Complete Genome Sequence of the California MSW Strain of Myxoma Virus Reveals Potential Host Adaptations. J Virol. 87(22):12080–12089. doi:10.1128/jvi.01923-13.

Kinnear M, Linde CC. 2010. Capsid gene divergence in rabbit hemorrhagic disease virus. J Gen Virol. 91(1):174–181. doi:10.1099/vir.0.014076-0.

Knight KL, Winsteadt CR. 1997. Generation of antibody diversity in rabbits. Curr Opin Immunol. 9:228–232.

Kolodziejek J, Huemer HP. 2010. Natural zoonotic infections of two marmosets and one

domestic rabbit with herpes simplex virus type 1 did not reveal a correlation with a certain gG- , gI- or gE genotype. doi:10.1111/j.1469-0691.2010.03163.x.

König M, Thiel HJ, Meyers G. 1998. Detection of viral proteins after infection of cultured hepatocytes with rabbit hemorrhagic disease virus. J Virol. 72(5):4492–4497. doi:10.1128/JVI.72.5.4492-4497.1998.

Kovaliski J. 1998. MONITORING THE SPREAD OF RABBIT HEMORRHAGIC DISEASE VIRUS AS A NEW BIOLOGICAL AGENT FOR CONTROL OF WILD EUROPEAN RABBITS IN AUSTRALIA Author (s): John Kovaliski EUROPEAN clinical. J Wildl Dis. 34(3):421–428. doi:http://dx.doi.org/10.7589/0090-3558-34.3.421.

Langenberg A, Smith D, Brakel CL, Pollice M, Remington M, Winter C, Dunne A, Corey L. 1988. Detection of herpes simplex virus DNA from genital lesions by in situ hybridization. J Clin Microbiol. 26(5):933–937. doi:10.1128/jcm.26.5.933-937.1988.

Laurent S, Vautherot JF, Madelaine MF, Le Gall G, Rasschaert D. 1994. Recombinant rabbit hemorrhagic disease virus capsid protein expressed in baculovirus self-assembles into viruslike particles and induces protection. J Virol. 68(10):6794–6798.

Lavazza A, Scicluna MT, Capucci L. 1996. Susceptibility of hares and rabbits to the European brown hare syndrome virus (EBHSV) and rabbit haemorrhagic disease virus (RHDV) under experimental conditions. Zentralbl Vet B. 43(7):401–410. doi:10.1111/j.1439-0450.1996.tb00332.x. http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citati on&list_uids=8885705.

Lavazza A, Tittarelli C, Cerioli M. 2015. The use of convalescent sera in immune-electron microscopy to detect non-suspected/new viral agents. Viruses. 7(5):2683–2703. doi:10.3390/v7052683.

Liu F, Zhou ZH. 2007. Comparative virion structures of human herpesviruses. In: Arvin A, Campadelli-Fiume G, Mocarski E, Moore PS, Roizman B, Whitley R, Yamanishi K, editors. Cambridge.

Liu G, Ni Z, Yun T, Yu B, Chen L, Zhao W, Hua J, Chen J. 2008. A DNA-launched reverse genetics system for rabbit hemorrhagic disease virus reveals that the VP2 protein is not essential for virus infectivity. J Gen Virol. 89(12):3080–3085. doi:10.1099/vir.0.2008/003525-0.

Liu S, Xue H, Pu B, Qian N. 1984. A new viral disease in rabbit. Anim Husb Vet Med. 16:253–255.

Liu W, Dang R, Wand X. 2015. Development of a SYBR-based real-time PCR to detect rabbit hemorrhagic disease virus (RHDV) and analyze its tissue distribution in experimentally infected rabbits. Virol Sin. 30(3):228–230. doi:10.1007/s12250-015-3560-0.

Lloyd HG. 1970. Post-myxomatosis rabbit populations in England and Wales. EPPO Public Ser A.(58):197–215.

Lopes AM, Blanco-Aguiar J, Martín-Alonso A, Leitão M, Foronda P, Mendes M, Gonçalves D, Abrantes J, Esteves PJ. 2018. Full genome sequences are key to disclose RHDV2 emergence in the Macaronesian islands. Virus Genes. 54(1):1–4. doi:10.1007/s11262-017-1523-2.

Lopes AM, Capucci L, Gavier-widén D, Gall-reculé G Le, Brocchi E, Barbieri I, Quéméner A, Le J, Geoghegan JL, Holmes EC, et al. 2014. Molecular evolution and antigenic variation of European brown hare syndrome virus (EBHSV). Virology. 468–470:104–112. doi:10.1016/j.virol.2014.08.002. http://dx.doi.org/10.1016/j.virol.2014.08.002.

Lopes AM, Correia J, Abrantes J, Melo P, Ramada M, Magalhães MJ, Alves PC, Esteves PJ. 2014. Is the new variant RHDV replacing genogroup 1 in portuguese wild rabbit populations? Viruses. 7(1):27–36. doi:10.3390/v7010027.

Lopes AM, Dalton KP, Magalha MJ, Parra F, Esteves PJ, Holmes EC, Abrantes J. 2015. Full genomic analysis of new variant rabbit hemorrhagic disease virus revealed multiple recombination events. :1309–1319. doi:10.1099/vir.0.000070.

Lopez-Martinez N. 2008. The Lagomorph fóssil record and origin of the European rabbit. In: Alves PC, Ferrand N, Hackländer K (Eds). Lagomorph Biology- Evolution, Ecology and Conservation. Springer-V. p. 27–46.

Luque D, Gonzalez JM, Gomez-Blanco J, Marabini R, Chichon J, Mena I, Angulo I, Carrascosa JL, Verdaguer N, Trus BL, et al. 2012. Epitope Insertion at the N-Terminal Molecular Switch of the Rabbit Hemorrhagic Disease Virus T=3 Capsid Protein Leads to Larger T=4 Capsids. J Virol. 86(12):6470–6480. doi:10.1128/JVI.07050-11. http://jvi.asm.org/cgi/doi/10.1128/JVI.07050-11.

Machín A, Martín Alonso JM, Parra F. 2001. Identification of the Amino Acid Residue Involved in Rabbit Hemorrhagic Disease Virus VPg Uridylylation. J Biol Chem. 276(30):27787–27792. doi:10.1074/jbc.M100707200.

Mahar JE, Hall RN, Peacock D, Kovaliski J, Piper M, Mourant R, Huang N. 2018. Rabbit Hemorrhagic Disease Virus 2 (RHDV2; GI.2) Is Replacing Endemic Strains of RHDV in the Australian Landscape within 18 Months of Its Arrival. J Virol. 2:1–15. doi:https://doi.org/10.1128/JVI.01374-17. Editor.

Mahmood T, Yang P-C. 2012. Western blot: technique, theory, and trouble shooting. N Am J Med Sci. 4(9):429–434. doi:10.4103/1947-2714.100998.

Marcato PS, Benazzi C, Vecchi G, Galeotti M, Della Salda L, Sarli G, Lucidi P. 1991. Clinical and pathological features of viral haemorrhagic disease of rabbits and the European brown hare syndrome. Rev Sci Tech. 10(2):371–392. doi:10.20506/rst.10.2.560.

Marcato PS, Rosmini R. 1986. Patologia del coniglio e della lepre, atlante a colori e compendio. Societ Edi. Bologna, Italy.

Marlier D. 2010. Vaccination strategies against myxomavirus infections: are we really doing the best? Tijdschr Diergeneeskd. 135(5):194–198.

Marlier D, Mainil J, Sulon J, Beckers JF, Linden A, Vindevogel H. 2000. Study of the virulence of five strains of amyxomatous myxoma virus in crossbred New Zealand White/Californian conventional rabbits, with evidence of long-term testicular infection in recovered animals. J Comp Pathol. 122(2–3):101–113. doi:10.1053/jcpa.1999.0345.

Marques RM, Costa-E-Silva A, Águas AP, Teixeira L, Ferreira PG. 2010. Early acute depletion of lymphocytes in calicivirus-infected adult rabbits. Vet Res Commun. 34(8):659–668. doi:10.1007/s11259-010-9437-7.

Marti JM, Biologi D De. 1999. Immunization with Potato Plants Expressing VP60 Protein

Protects against Rabbit Hemorrhagic Disease Virus. 73(5):4452–4455.

Martin-Alonso A, Martin-Carrillo N, Garcia-Livia K, Valladares B, Foronda P. 2016. Emerging rabbit haemorrhagic disease virus 2 (RHDV2) at the gates of the African continent. Infect Genet Evol. 44:46–50. doi:10.1016/j.meegid.2016.06.034. http://dx.doi.org/10.1016/j.meegid.2016.06.034.

Martín Alonso JM, Casais R, Boga J a, Parra F. 1996. Processing of rabbit hemorrhagic disease virus polyprotein. J Virol. 70(2):1261–1265.

Martin SCJ. 1936. Observations on myxomatosis cuniculi (Sanarelli) made with a view to the use of the virus in the control of rabbit plagues. Government Printer, South Africa.

McColl KA, Morrissy CJ, Collins BJ, Westbury HA. 2002. Persistence of rabbit haemorrhagic disease virus in decomposing rabbit carcases. Aust Vet J. 80:298–299.

McCombs R, Brunschwig J, Mirkovic R. 1971. Electron microscopic characterization of a herpeslike virus isolated from tree shrews. Virology. 45:816–820.

McIntosh MT, Behan SC, Mohamed FM, Lu Z, Moran KE, Burrage TG, Neilan JG, Ward GB, Botti G, Capucci L, et al. 2007. A pandemic strain of calicivirus threatens rabbit industries in the Americas. Virol J. 4:96. doi:10.1186/1743-422X-4-96. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2147015&tool=pmcentrez&rendertype=abstract.

Médail F, Quézel P. 1999. Biodiversity Hotspots in the Mediterranean Basin : Setting Global Conservation Priorities. 13(6):1510–1513.

Medveczky PG. 1999. Herpesvirus sylvilagus (Herpesviridae). In: Allan G, Robert GW, editors. Encyclopedia of virology. Oxford (United Kingdom): Elsevier. p. 703–6.

Merchán T, Rocha G, Alda F, Silva E, Thompson G, de Trucios SH, Pagés A. 2011. Detection of rabbit haemorrhagic disease virus (RHDV) in nonspecific vertebrate hosts sympatric to the European wild rabbit (Oryctolagus cuniculus). Infect Genet Evol. 11(6):1469–1474. doi:10.1016/j.meegid.2011.05.001.

Mettenleiter TC. 2006. Intriguing interplay between viral proteins during herpesvirus assembly or: the herpesvirus assembly puzzle. Vet Microbiol. 113(3–4):163–169. doi:10.1016/j.vetmic.2005.11.040.

Meyers G, Tu D-. 2003. Translation of the Minor Capsid Protein of a Calicivirus Is Initiated by a Novel Termination-dependent Reinitiation Mechanism * □. J Biol Chem. 278(36):34051–34060. doi:10.1074/jbc.M304874200.

Meyers G, Wirblich C, Thiel H-J, Thumfart JO. 2000. Rabbit Hemorrhagic Disease Virus: Genome Organization and Polyprotein Processing of a Calicivirus Studied after Transient Expression of cDNA Constructs. Virology. 276(2):349–363. doi:10.1006/viro.2000.0545. http://linkinghub.elsevier.com/retrieve/pii/S0042682200905452.

Meyers G, Wirblich C, Thiel HJ. 1991. Rabbit hemorrhagic disease virus-molecular cloning and nucleotide sequencing of a calicivirus genome. Virology. 184(2):664–676. doi:10.1016/0042-6822(91)90436-F.

Michael K, Klupp BG, Mettenleiter TC, Karger A. 2006. Composition of pseudorabies virus particles lacking tegument protein US3, UL47, or UL49 or envelope glycoprotein

E. J Virol. 80(3):1332–1339. doi:10.1128/JVI.80.3.1332-1339.2006.

Micozzi G, Palarchi M. 1965. Spontaneous myxomatosis in hares. Vet Ital. 26:361–362.

Miller WA, Koev G. 2000. Synthesis of Subgenomic RNAs by Positive-Strand RNA Viruses. Virology. 273(1):1–8. doi:10.1006/viro.2000.0421. http://linkinghub.elsevier.com/retrieve/pii/S0042682200904215.

Mittermeier RA, Gil PR, Hoffman M, Pilgrim J, Brooks T, Mittermeier CG, Lamoreux J, da Fonseca GAB. 2005. Hotspots revisited: earth's biologically richest and most endangered terrestrial ecoregions. Conserv Int Mex City.

Mohsen MO, Zha L, Cabral-miranda G, Bachmann MF. 2017. Seminars in Immunology Major fi ndings and recent advances in virus – like particle (VLP) -based vaccines. Semin Immunol. 34(August):123–132. doi:10.1016/j.smim.2017.08.014. https://doi.org/10.1016/j.smim.2017.08.014.

Monnerot M, Vigne J, Biju-Duval C, Casane D, Callou C, Hardy C, Mougel F, Soriguer R, Dennebouy N, Mounolou J. 1994. Rabbit and man: genetic and historic approach. enet Sel Evol 26, S167. doi:10.1186/1297-9686-26-S1-S167.

Monterroso P, Garrote G, Serronha A, Santos E, Delibes-Mateos M, Abrantes J, Perez de Ayala R, Silvestre F, Carvalho J, Vasco I, et al. 2016. Disease-mediated bottom-up regulation: An emergent virus affects a keystone prey, and alters the dynamics of trophic webs. Sci Rep. 6(1):36072. doi:10.1038/srep36072. http://www.nature.com/articles/srep36072.

Morales M, Ramírez MA, Cano MJ, Párraga M, Castilla J, Pérez-Ordoyo LI, Torres JM, Bárcena J. 2009. Genome Comparison of a Nonpathogenic Myxoma Virus Field Strain with Its Ancestor, the Virulent Lausanne Strain. J Virol. 83(5):2397–2403. doi:10.1128/jvi.02189-08.

Morisse J, Le Gall G, Boilletot E. 1991. Hepatitis of viral origin in Leporidae: introduction and aetiological hypotheses. Rev Sci Tech. 10:283–295.

Moss SR, Turner SL, Trout RC, White PJ, Hudson PJ, Desai A, Armesto M, Forrester NL, Gould EA. 2002. Molecular epidemiology of Rabbit haemorrhagic disease virus. :2461–2467.

Muller A, Silva E, Abrantes J, Esteves PJ, Ferreira PG, Carvalheira JC, Nowotny N, Thompson G. 2010. Partial sequencing of recent Portuguese myxoma virus field isolates exhibits a high degree of genetic stability. Vet Microbiol. 140(1–2):161–166.

Müller C, Hrynkiewicz R, Dominika B, Maldonado J, Köllner B, Nied P. 2021. Immunity against Lagovirus europaeus and the Impact of the Immunological Studies on Vaccination. :1–25.

Müller C, Ulrich R, Schinköthe J, Müller M, Köllner B. 2019. Characterization of protective humoral and cellular immune responses against RHDV2 induced by a new vaccine based on recombinant baculovirus. 37:4195–4203. doi:10.1016/j.vaccine.2019.04.061.

Muller K, Fuchs W, Heblinski N, Teifke JP, Brunnberg L, Gruber AD, Klopfleisch R. 2009. Encephalitis in a rabbit caused by human herpesvirus-1. 235(1).

Myers N, Mittermeier RA, Mittermeier CG, Fonseca GAB, Kent J. 2000. Biodiversity hotspots for conservation priorities. Nature. 403(February):853–858.

Nagesha HS, McColl KA, Collins BJ, Morrissy CJ, Wang LF, Westbury HA. 2000. The presence of cross-reactive antibodies to rabbit haemorrhagic disease virus in Australian wild rabbits prior to the escape of virus from quarantine. Arch Virol. 145(4):749–757. http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citati on&list_uids=10893153.

Nagesha HS, Wang LF, Hyatt AD, Morrissy CJ, Lenghaus C, Westbury HA. 1995. Self-assembly, antigenicity, and immunogenicity of the rabbit haemorrhagic disease virus (Czechoslovakian strain V-351) capsid protein expressed in baculovirus. Arch Virol. 140:1095–1108.

Neave MJ, Hall RN, Huang N, Mccoll KA, Kerr P, Hoehn M, Taylor J, Strive T. 2018. Robust Innate Immunity of Young Rabbits Mediates Resistance to Rabbit Hemorrhagic Disease Caused. Viruses. 10:1–22. doi:10.3390/v10090512.

Neimanis Aleksija S, Ahola H, Pettersson UL, Lopes AM, Abrantes J, Zohari S, Esteves PJ, Gavier-widén D. 2018. Overcoming species barriers : an outbreak of Lagovirus europaeus GI . 2 / RHDV2 in an isolated population of mountain hares (Lepus timidus). :1–12.

Neimanis A S, Ahola H, Zohari S, Larsson Pettersson U, Bröjer C, Capucci L, Gavier-Widén D. 2018. Arrival of rabbit haemorrhagic disease virus 2 to northern Europe: Emergence and outbreaks in wild and domestic rabbits (Oryctolagus cuniculus) in Sweden. Transbound Emerg Dis. 65(1):213–220. doi:10.1111/tbed.12650.

Niedźwiedzka-rystwej P, Deptuła W. 2012. Apoptosis of peripheral blood leukocytes from rabbits infected with non-haemagglutinating strains of rabbit haemorrhagic disease virus (RHDV). Vet Immunol Immunopathol. 149:54–57. doi:10.1016/j.vetimm.2012.06.012.

Niedźwiedzka-Rystwej P, Deptuła W. 2010. Cytometric analysis of lymphocytes T and B in rabbits infected with non-haemagglutinogenic strains of RHD virus (rabbit haemorrhagic disease)--Rainham, Frankfurt and Asturias. Pol J Vet Sci. 13(1):157–162.

Niedźwiedzka-Rystwej P, Hukowska-szematowicz B, Tokarz-deptuła B, Trzeciak-ryczek A, Działo J, Deptuła W. 2013. Apoptosis of peripheral blood leucocytes in rabbits infected with different strains of rabbit haemorrhagic disease virus. 60(1):65–69.

Niedźwiedzka-Rystwej P., Tokarz-Deptula B, Deptula W. 2013. Lymphocytes T and B in rabbits infected with RHD virus. Pol J Vet Sci. 16(3):563–565. doi:10.2478/pjvs-2013-0077.

Nowotny N, Bascun CR. 1997. Phylogenetic analysis of rabbit haemorrhagic disease and European brown hare syndrome viruses by comparison of sequences from the capsid protein gene. :657–673.

Nowotny N, Fuchs A, Schilcher F, Loupal G. 1990. Occurrence of viral haemorrhagic disease of rabbits in Austria. Pathol Virol Investig [in Ger. 77(Wiener Tierärztliche Monatsschrift):19–23.

Nyström K, Le Gall-Reculé G, Grassi P, Abrantes J, Ruvoën-Clouet N, Le Moullac-Vaidye B, Lopes AM, Esteves PJ, Strive T, Marchandeau S, et al. 2011. Histo-blood group antigens act as attachment factors of rabbit hemorrhagic disease virus infection in a virus strain-dependent manner. PLoS Pathog. 7(8). doi:10.1371/journal.ppat.1002188.

O'Connor CM, Kedes DH. 2006. Mass spectrometric analyses of purified rhesus monkey rhadinovirus reveal 33 virion-associated proteins. J Virol. 80(3):1574–1583. doi:10.1128/JVI.80.3.1574-1583.2006.

Ohlinger VF, Haas B, Meyers G, Weiland F, Thiel H-J. 1990. Identification and Characterization of the Virus Causing Rabbit Hemorrhagic Disease. J Virol. 64(7):3331–3336.

OIE. 2018. Rabbit Haemorrhagic Disease. In: OIE Terrestrial Manual 2018. p. 1389–1406.

OIE. 2019. Rabbit haemorrhagic disease (Technical disease card). :1–7. [accessed 2020 Aug 1]. https://www.oie.int/standard-setting/terrestrial-manual/.

OIE. 2020. Myxomatosis. [accessed 2020 May 26]. https://www.oie.int/en/animal-health-in-the-world/animal-diseases/Myxomatosis/.

Onderka DK, Papp-vid G, Perry AW. 1992. Fatal herpesvirus infection in commercial rabbits. 33(August).

Parra F, Boga JA, Marin MS, Casais R. 1993. The amino terminal sequence of VP60 from rabbit hemorrhagic disease virus supports its putative subgenomic origin. Virus Res. 27(3):219–228. doi:10.1016/0168-1702(93)90034-k.

Parra F, Prieto M. 1990. Purification and Characterization of a Calicivirus as the Causative Agent of a Lethal Hemorrhagic Disease in Rabbits. J Virol. 64(8):4013–4015.

Pellett P, Davison A, Eberle R. 2012. Order Herpesvirales. In: King AMQ, Adams MJ, Carstens EB, Lefkowitz EJ, editors. Virus Taxonomy: Ninth report of the International Committee on Taxonomy of Viruses. Oxford: Elsevier. p. 99–107.

Pellett PE, Roizman B. 2013. Herpesviridae. In: Knipe DM, Howley PM, editors. Fields Virology. 6th ed. Philadelphia, PA 19103 USA: Wolters Kluwer. p. 1802–1822.

Peñaflor-téllez Y, Trujillo-uscanga A, Escobar-almazán JA, Gutiérrez-escolano AL. 2019. Immune Response Modulation by Caliciviruses. 10(October):1–14. doi:10.3389/fimmu.2019.02334.

Le Pendu J, Abrantes J, Bertagnoli S, Guitton J-S, Le Gall-Reculé G, Lopes AM, Marchandeau S, Alda F, Almeida T, Célio AP, et al. 2017. Proposal for a unified classification system and nomenclature of lagoviruses. J Gen Virol. 98(7):1658—1666. doi:10.1099/jgv.0.000840. https://doi.org/10.1099/jgv.0.000840.

Pinto A, Matos AL De, Abrantes M, Kraberger S, Risalde MA, Gort C, Mcfadden G, Varsani A, Esteves PJ. 2019. Genetic Characterization of a Recombinant Myxoma Virus in the Iberian Hare (Lepus granatensis). Viruses. 11(530):1–16. doi:10.3390/v11060530.

Prepens S, Kreuzer K, Leendertz F, Nitsche A, Ehlers B. 2007. Discovery of herpesviruses in multi-infected primates using locked nucleic acids (LNA) and a bigenic PCR approach. 14:1–14. doi:10.1186/1743-422X-4-84.

Puggioni G, Cavadini P, Maestrale C, Scivoli R, Botti G, Ligios C, Le Gall-Reculé G, Lavazza A, Capucci L. 2013. The new French 2010 Rabbit Hemorrhagic Disease Virus causes an RHD-like disease in the Sardinian Cape hare (Lepus capensis mediterraneus). Vet Res. 44(1):1–7. doi:10.1186/1297-9716-44-96.

Ratcliffe FN, Myers K, Fennessy B V, Calaby JH. 1952. Myxomatosis in Australia: a step towards the biological control of the rabbit. Nature. 170(4314):7–11.

Rivers TM. 1930. INFECTIOUS MYXOMATOSIS OF RABBITS. J Exper Med. 51(965).

Robinson AJ, Kirkland PD, Forrester RI, Capucci L, Cooke BD, Philbey AW. 2002. Serological evidence for the presence of a calicivirus in Australian wild rabbits, Oryctolagus cuniculus, before the introduction of rabbit haemorrhagic disease virus (RHDV): its potential influence on the specificity of a competitive ELISA for RHDV A. Wildl Res. 29:655–662.

Rogers PM, Soriguer RC, Arthur CP. 1994. The rabbit in continental Europe.

Roizmanm B, Furlong D. 1974. The replication of herpesviruses. In: Fraenkel- Conrat H, editor. Comprehensive Virology. New York: Plenum Press; 229–403. p. 229–403.

Rose TM, Schultz ER, Henikoff JG, Pietrokovski S, Mccallum CM, Henikoff S. 1998. Consensus-degenerate hybrid oligonucleotide primers for amplification of distantly related sequences. 26(7):1628–1635.

Rouco C, Aguayo-adán JA, Santoro S, Abrantes J, Delibes-mateos M. 2019. Worldwide rapid spread of the novel rabbit haemorrhagic disease virus (GI.2/RHDV2/b). Transbound Emerg Dis. 66(4):1762–4. doi:10.1111/tbed.13189.

Ruvoën-Clouet N, Ganiere JP, Andre-Fontaine G, Blanchard D, Le Pendu J. 2000. Binding of Rabbit Hemorrhagic Disease Virus to Antigens of the ABH Histo-Blood Group Family. J Virol. 74(24):11950–11954. doi:10.1128/JVI.74.24.11950-11954.2000. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=112478&tool=pmcentrez&rendertype=abstract%5Cnhttp://jvi.asm.org/cgi/doi/10.1128/JVI.74.24.11950-11954.2000.

Saint KM, French N, Kerr P. 2001. Genetic variation in Australian isolates of myxoma virus: an evolutionary and epidemiological study. Arch Virol. 146(6):1105–1123.

Saito JK, McKercher DG, Castrucci G. 1964. Attenuation of the Myxoma Virus and Use of the Living Attenuated Virus as an Immunizing Agent for Myxomatosis. J Infect Dis. 114(5):417–428. doi:10.1093/infdis/114.5.417. https://doi.org/10.1093/infdis/114.5.417.

Sanarelli G. 1898. Das myxomatogene Virus. Beitrag zum Studer Krankheitserreger ausserhalb des sichtbaren. Cent f Bakt,. Abt. I.(23):865.

Sanchez-Campos S, Alvarez M, Culebras JM, Gonzalez-Gallego J, Tunon MJ. 2004. Pathogenic molecular mechanisms in an animal model of fulminant hepatic failure: Rabbit hemorrhagic viral disease. J Lab Clin Med. 144(4):215–222. doi:10.1016/j.lab.2004.07.006.

Schirrmeier H, Reimann I, Granzow H, Riems I. 1999. Pathogenic , antigenic and molecular properties of rabbit haemorrhagic disease virus (RHDV) isolated from vaccinated rabbits : detection and characterization of antigenic variants. :719–735.

Schramlová J, Arientová S, Hulínská D. 2010. The role of electron microscopy in the rapid diagnosis of viral infections--review. Folia Microbiol (Praha). 55(1):88–101. doi:10.1007/s12223-010-0015-8.

Semerjyan AB, Sargsyan MA, Arzumanyan HH, Hayrapet L. 2019. Immune cell pathology in rabbit hemorrhagic disease. 12:1332–1340. doi:10.14202/vetworld.2019.1332-1340.

Serradell MC, Rupil LL, Martino RA, Prucca CG, Carranza PG, Saura A, Fernández EA, Gargantini PR, Tenaglia AH, Petiti JP, et al. 2019. Efficient oral vaccination by bioengineering virus-like particles with protozoan surface proteins. Nat Commun. 10(361). doi:10.1038/s41467-018-08265-9. http://dx.doi.org/10.1038/s41467-018-08265-9.

Sibilia M, Boniotti MB, Angoscini P, Capucci L, Rossi C. 1995. Two independent pathways of expression lead to self-assembly of the rabbit hemorrhagic disease virus capsid protein. J Virol. 69(9):5812–5. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=189447&tool=pmcentrez&rendertype=abstract.

Silvério D, Lopes AM, Melo-ferreira J, Magalhaes MJ, Ponterroso P, Serronha A, Maio E, Alves PC, Esteves PJ, Abrantes J. 2018. Insights into the evolution of the new variant rabbit haemorrhagic disease virus (GI . 2) and the identification of novel recombinant strains. Transbound Emerg Dis. 00:1–10. doi:10.1111/tbed.12830.

Singh A, Frcpc B, Frcpc JP, Frcpc BR. 2005. The laboratory diagnosis of herpes simplex virus infections. Can J Infect Dis Med Microbiol. 16(2):92–98.

Šmíd B, Valíček L, Rodák L, Štěpánek J, Jurák E. 1991. Rabbit haemorrhagic disease: an investigation of some properties of the virus and evaluation of an inactivated vaccine. Vet Microbiol. 26(1–2):77–85. doi:10.1016/0378-1135(91)90043-F.

Smid B, ValiOek L, Roddk L, Jurdk E, Stepanek J. 1989. Virové hemorrhagické onemocneni krfilikfi (mor krfiliku), dosavadni poznatky a soueasnfi situace. Veterinarstvi. 39((in Czech.)):490–491.

Sobey WR, Conolly D, Adams KM. 1966. Myxomatosis: a simple method of sampling blood and testing for circulating soluble antigens or antibodies to them. Aust J Sci. 28:354.

Sobey WR, Menzies W. 1969. Myxomatosis: the introduction of the European rabbit flea Spilosyllus cuniculi (Dale) into Australia. Aust J Sci.

Soriguer RC. 1980. y curva de crecimiento. 7(1):83–90.

Spiesschaert B, Mcfadden G, Hermans K, Nauwynck H, Walle GR Van De. 2011. The current status and future directions of myxoma virus , a master in immune evasion. Vet Res. 42(1):76. doi:10.1186/1297-9716-42-76. http://www.veterinaryresearch.org/content/42/1/76.

Stoercklé-Berger N, Keller-Berger B, Ackermann M, Ehrensperger F. 1992. Immunohistological diagnosis of rabbit haemorrhagic disease (RHD). Zentralbl Veterinarmed B. 39(4):237–245. doi:10.1111/j.1439-0450.1992.tb01164.x.

Strive T, Elsworth P, Liu J, Wright JD, Kovaliski J, Capucci L. 2013. The non-pathogenic Australian rabbit calicivirus RCV-A1 provides temporal and partial cross protection to lethal Rabbit Haemorrhagic Disease Virus infection which is not dependent on antibody titres. Vet Res. 44(1):1–11. doi:10.1186/1297-9716-44-51.

Stuart Chapin F, Zavaleta ES, Eviner VT, Naylor RL, Vitousek PM, Reynolds HL, Hooper DU, Lavorel S, Sala OE, Hobbie SE, et al. 2000. Consequences of changing biodiversity. Nature. 405(May).

Swan C, Perry A, Papp-Vid G. 1991. Alberta. Herpesvirus-like viral infection in a rabbit.

Can Vet J = La Rev Vet Can. 32(10):627–628.

Szillat KP, Höper D, Beer M, König P. 2020. Full-genome sequencing of German rabbit haemorrhagic disease virus uncovers recombination between RHDV (GI.2) and EBHSV (GII.1). Virus Evol. 6(2). doi:10.1093/ve/veaa080. https://doi.org/10.1093/ve/veaa080.

Tatem AJ, Rogers DJ, Hay SI. 2006. Global Transport Networks and Infectious Disease Spread. 62(05). doi:10.1016/S0065-308X(05)62009-X.

Taube S, Jiang M, Wobus CE. 2010. Glycosphingolipids as receptors for non-enveloped viruses. Viruses. 2(4):1011–1049. doi:10.3390/v2041011.

Teixeira L, Marques RM, Águas AP, Ferreira PG. 2012. Veterinary Immunology and Immunopathology Regulatory T cells are decreased in acute RHDV lethal infection of adult rabbits. Vet Immunol Immunopathol. 148(3–4):343–347. doi:10.1016/j.vetimm.2012.05.006. http://dx.doi.org/10.1016/j.vetimm.2012.05.006.

Thackray LB, Wobus CE, Chachu KA, Liu B, Alegre ER, Henderson KS, Kelley ST, Virgin HW 4th. 2007. Murine noroviruses comprising a single genogroup exhibit biological diversity despite limited sequence divergence. J Virol. 81(19):10460–10473. doi:10.1128/JVI.00783-07.

Thompson HV, King CM. 1994. The European rabbit: the history and bioogy of a successful colonizer. Oxford Univ Press Oxford United Kingdom.

Thouvenin E, Laurent S, Madelaine MF, Rasschaert D, Vautherot JF, Hewat E a. 1997. Bivalent binding of a neutralising antibody to a calicivirus involves the torsional flexibility of the antibody hinge. J Mol Biol. 270(2):238–46. doi:10.1006/jmbi.1997.1095. http://www.ncbi.nlm.nih.gov/pubmed/9236125.

Trzeciak-Ryczek A, Tokarz-Deptu B. 2016. Expression of IL-1b, IL-2, IL-10, TNF-b and GM-CSF in peripheral blood leukocytes of rabbits experimentally infected with rabbit haemorrhagic disease virus. Vet Microbiol. 186:71–81. doi:10.1016/j.vetmic.2016.02.021.

Trzeciak-Ryczek A, Tokarz-Deptu B. 2017. Expression of IL-1Ra, IL-6, IL-8, IL-18, TNF-a and IFN-g genes in peripheral blood leukocytes of rabbits infected with RHDV (Rabbit Haemorrhagic Disease Virus). Dev Comp Immunol. 76:310–315. doi:10.1016/j.dci.2017.07.005.

Trzeciak-Ryczek A, Tokarz-Deptuła B, Deptuła W. 2015. The importance of liver lesions and changes to biochemical and coagulation factors in the pathogenesis of RHD. Acta Biochim Pol. 62(2):169–171. doi:10.18388/abp.2014_943.

Tunon MJ, San Miguel B, Crespo I, Riezu-Boj JI, Larrea E, Alvarez M, Gonzalez I, Bustos M, Gonzalez-Gallego J, Prieto J. 2011. Cardiotrophin-1 Promotes a High Survival Rate in Rabbits with Lethal Fulminant Hepatitis of Viral Origin. J Virol. 85(24):13124–13132. doi:10.1128/JVI.05725-11. http://jvi.asm.org/cgi/doi/10.1128/JVI.05725-11.

Ueda K, Park JH, Ochiai K, Itakura C. 1992. Disseminated intravascular coagulation (DIC) in rabbit haemorrhagic disease. Jpn J Vet Res. 40(4):133–141.

Ulsenheimer A, Gerlach JT, Jung M, Gruener N, Martin W, Backmund M, Santantonio T, Schraut W, Heeg MHJ, Schirren CA, et al. 2005. Plasmacytoid Dendritic Cells in Acute and Chronic Hepatitis C Virus Infection ¨. Hepatology. 41(3):643–651. doi:10.1002/hep.20592.

Upton C, Slack S, Hunter AL, Ehlers A, Roper RL. 2003. Poxvirus Orthologous Clusters: toward Defining the Minimum Essential Poxvirus Genome. J Virol. 77(13):7590–7600. doi:10.1128/jvi.77.13.7590-7600.2003.

Valicek L, Smid B, Rodak L, Kudrna J. 1990. Electron and immunoelectron microscopy of rabbit haemorrhagic disease virus (RHDV). Arch Virol. 112:71–275.

Varnum SM, Streblow DN, Monroe ME, Smith P, Auberry KJ, Pasa-Tolic L, Wang D, Camp DG 2nd, Rodland K, Wiley S, et al. 2004. Identification of proteins in human cytomegalovirus (HCMV) particles: the HCMV proteome. J Virol. 78(20):10960–10966. doi:10.1128/JVI.78.20.10960-10966.2004.

Velarde R, Abrantes J, Lopes AM, Estruch J, Côrte-Real J V., Esteves PJ, García-Bocanegra I, Ruiz-Olmo J, Rouco C. 2021. Spillover event of recombinant Lagovirus europaeus/GI.2 into the Iberian hare (Lepus granatensis) in Spain. Transbound Emerg Dis.(May):1–7. doi:10.1111/tbed.14264.

Velarde R, Cavadini P, Neimanis A, Cabezón O, Chiari M, Gaffuri A, Lavín S, Grilli G, Gavier-Widén D, Lavazza A, et al. 2017. Spillover Events of Infection of Brown Hares (Lepus europaeus) with Rabbit Haemorrhagic Disease Type 2 Virus (RHDV2) Caused Sporadic Cases of an European Brown Hare Syndrome-Like Disease in Italy and Spain. Transbound Emerg Dis. 64(6):1750–1761. doi:10.1111/tbed.12562.

Viaplana E, Plana J, Villaverde A. 1997. Antigenicity of VP60 structural protein of rabbit haemorrhagic disease virus. Arch Virol. 142(9):1843–1848. doi:10.1007/s007050050201.

Villafuerte R, Calvete C, Blanco JC, Lucientes J. 1995. Incidence of viral hemorrhagic disease in wild rabbit populations in Spain. Mammalia.:651–660. doi:https://doi.org/10.1515/mamm.1995.59.4.651.

Villafuerte R, Delibes-Mateos M. 2019. Oryctolagus cuniculus (errata version published in 2020). IUCN Red List Threat Species 2019 eT41291A170619657. doi:https://dx.doi.org/10.2305/IUCN.UK.2019-3.RLTS.T41291A170619657.en.

Vinjé J, Estes MK, Esteves P, Green KY, Katayama K, Knowles NJ, L'Homme Y, Martella V, Vennema H, White PA, et al. 2019. ICTV Virus Taxonomy Profile: Caliciviridae. J Gen Virol. 100(11):1469–1470. doi:https://doi.org/10.1099/jgv.0.001332. https://www.microbiologyresearch.org/content/journal/jgv/10.1099/jgv.0.001332.

Vitousek PM, Antonio CMD, Loope LL, Rejmánek M, Westbrooks R, Carolina N. 2015. New Zealand Ecological Society INTRODUCED SPECIES : A SIGNIFICANT COMPONENT OF HUMAN-CAUSED GLOBAL CHANGE Linked references are available on JSTOR for this article : All use subject to JSTOR Terms and Conditions SPECIES : A SIGNIFICANT INTRODUCED HUMAN-CA. 21(1):1–16.

Van der Wal R, Wijnen HV, Wieren SV, Beucher O, Bos D. 2000. ON FACILITATION BETWEEN HERBIVORES : HOW BRENT GEESE PROFIT. Ecology. 81(4):969–980.

Ward D. 2005. Reversing Rabbit Decline: One of the biggest challenges for nature conservation in Spain and Portugal. SOSLynx.opg.

Weissenbock H, Hainfellner JA, Berger J, Kasper I, Budka H. 1997. Naturally ocurring Herpes Simplex encephalitis in a Domestic Rabbit (Oryctolagus cuniculus). Vet Pathol. 34:44–47.

Westcott DG, Frossard J-P, Everest D, Dastjerdi A, Duff JP, Choudhury B. 2014. Incursion of RHDV2-like variant in Great Britain. Vet Rec. 174(13):333.1-333. doi:10.1136/vr.g2345. http://veterinaryrecord.bmj.com/lookup/doi/10.1136/vr.g2345.

Wiedbrauk D, Johnston S. 1993. Manual of Clinical Virology. :109–20.

Wilks AF, Kurban RR, Hovens CM, Ralph SJ. 1989. The application of the polymerase chain reaction to cloning members of the protein tyrosine kinase family. Gene. 85(1):67–74. doi:10.1016/0378-1119(89)90465-4.

Willer DO, McFadden G, Evans DH. 1999. The complete genome sequence of shope (rabbit) fibroma virus. Virology. 264(2):319–343.

Williams CK, Parer I, Coman BJ, Burley J, Braysher ML. 1995. Managing vertebrate pests: rabbits.

Wirblich C, Meyers G, Ohlinger VF, Capucci L, Eskens U, Haas B, Thiel HJ. 1994. European brown hare syndrome virus: relationship to rabbit hemorrhagic disease virus and other caliciviruses. J Virol. 68(8):5164–73. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=236460&tool=pmcentrez&rendertype=abstract.

Wirblich C, Thiel HJ, Meyers G. 1996. Genetic map of the calicivirus rabbit hemorrhagic disease virus as deduced from in vitro translation studies. J Virol. 70(11):7974–83. doi:10.1006/viro.2000.0579. http://jvi.asm.org/content/70/11/7974.short%5Cnhttp://www.ncbi.nlm.nih.gov/pubmed/8892921%5Cnhttp://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC190870%5Cnhttp://www.ncbi.nlm.nih.gov/pubmed/11062050%5Cnhttp://linkinghub.elsevier.com/retrieve/pii/.

WWF. 2020. Living Planet Report 2020 - Bending the curve of biodiversity loss. Almond REA, M. G, Petersen T, editors. WWF, Gland, Switzerland.

Xu W. 1991. Viral haemorrhagic disease of rabbits in the People's Republic of China: epidemiology and virus characterisation. Rev Sci Tech.:10: 393-408.

Xu ZJ, Chen WX. 1989. Viral haemorrhagic disease in rabbits: A review. Vet Res Commun. 13(3):205–212. doi:10.1007/BF00142046.

Yang HC. 1989. A study on detecting RHD antibodies by the indirect haemagglutination test (in Chinese). Anim Hus vet Med. 21(3):100–10.

Yang HC, Xu Y, Du NX. 1989. Studies on the haemagglutination characteristics of virus of rabbit haemorrhagic disease (in Chinese). Anim Hus vet Med. 21(1):23–26.

Yuan D, Guo D, Liu J, Si C, Jiang Q, Lin H, Yang T, Qu L. 2013. Development of a reverse-transcription loop-mediated isothermal amplification method for detection of rabbit hemorrhagic disease virus. J Virol Methods. 187(2):274–277. doi:10.1016/j.jviromet.2012.11.020.

Zepeda-Cervantes J, Ramírez-jarquín JO, Vaca L. 2020. Interaction Between Virus-Like Particles (VLPs) and Pattern Recognition Receptors (PRRs) From Dendritic Cells (DCs): Toward Better Engineering of VLPs. 11(June):1–22. doi:10.3389/fimmu.2020.01100.

Zygraich N, Berge E. 1972. Experimental Infection of Rabbits and Monkeys with Herpesvirus cuniculi. 13:241–244.

Publication impact factor report

These 14 studies are published in international peer-reviewed, listed below by the order that appear in the document.

Estimated percentage of contribution for bench/ first writing work	Relative Classification	Reference
90%	Q2 I.F. 1.474 5-year I.F. 1.495	**Abade dos Santos, F.A.**; Carvalho, C.L.; Peleteiro, M.C.; Gabriel, S.;.I.; Patrício, R.; et al. Blood collection from the external jugular vein of *Oryctolagus cuniculus algirus* sedated with midazolam : live sampling of a subspecies at risk *Wildlife Biol.* **2019**, *1*, doi:10.2981/wlb.00588.
95%	Q1 I.F. 4.326	**Abade dos Santos, F.A.**; Carvalho, C.L.; Almeida, I.; Fagulha, T.; Ramos, F.; Barros, S.C.; Henriques, M.; Luís, T.; Duarte, M.D. Simple Method for Establishing Primary Leporidae Skin Fibroblast Cultures. Cells 2021, 10, 1–9, doi:https://doi.org/10.3390/cells10082100:
40%	Q1 I.F. 5.640 5-year I.F. 5.489	**Abade dos Santos, F.A.**; Portela, S.J.; Nogueira, T.; Carvalho, C.L.; de Sousa, R.; Duarte, M.D. Harmless or Threatening? Interpreting the Results of Molecular Diagnosis in the Context of Virus-Host Relationships. Front. Microbiol. 2021, 12, 1–8, doi:10.3389/fmicb.2021.647730.
95%	Q1 I.F. 5.542 5-year I.F. 5.708	**Abade dos Santos, F.A.**; Carvalho, C.L.; Parra, F.; Dalton, K.P.; Peleteiro, M.C. Duarte, M.D. A Quadruplex qPCR for Detection and Differentiation of Classic and Natural Recombinant Myxoma Virus Strains of Leporids. Int. J. Mol. Sci. 2021, 1–19.
95%	Q1 I.F. 4.91 5-year I.F. 4.975	**Abade dos Santos, F.A.**; Carvalho, C.L.; Peleteiro, M.C.; Parra, F.; Duarte, M.D. A Versatile qPCR for Diagnosis of Leporid Gammaherpesvirus 5 Using Evagreen® or Taqman® Technologies. *Viruses* **2021**, *13*, doi:10.3390/v13040715.

80%	Q1 I.F. 5.005 5-year I.F. 4.761	**Abade dos Santos, F.A.**; Pinto, A.; Burgoyne, T.; Dalton, K.P.; Carvalho, C.L.; Ramilo, D.W.; Carneiro, C.; Carvalho, T.; Peleteiro, M.C.; Parra, F.; et al. Spillover events of Rabbit Hemorrhagic Disease Virus 2 (recombinant GI.4P-GI.2) from Lagomorpha to Eurasian badger. Transbound. Emerg. Dis. 2021, 1–16, doi:10.1111/tbed.14059.
75%	Q1 I.F. 2.752 5-year I.F. 2.739	**Abade dos Santos, F.A.;** Magro, C.; Carvalho, C.L.; Ruivo, P.; Duarte, M.D.; Peleteiro, M.C. A Potential Atypical Case of Rabbit Haemorrhagic Disease in a Dwarf Rabbit. Animals 2021, 11, doi:https://doi.org/10.3390/ani11010040.
20%	Q4 I.F. 0.221 5-year I.F. 0.236	Carvalho, C.L.**; Abade dos Santos, F.A**.; Fagulha, T.; Carvalho, P.; Mendonça, P.; Monteiro, M.; Duarte, M.D. Myxoma virus and rabbit haemorrhagic disease virus 2 coinfection in a European wild rabbit (*Oryctolagus cuniculus algirus*), Portugal. 2020, 18–21, doi:10.1136/vetreccr-2019-001002.
20%	Q4 I.F. 0.221 5-year I.F. 0.236	Carvalho, C.; **Abade Dos Santos, F.A.**; Monteiro, M.; Carvalho, P.; Mendonça, P.; Duarte, M.D. First cases of myxomatosis in Iberian hares (Lepus granatensis) in Portugal. Vet. Rec. Case Reports 2020, 8, doi:10.1136/vetreccr-2019-001044.
90%	Q1 I.F. 4.91 5-year I.F. 4.975	**Abade dos Santos, F.A**.; Carvalho, C.; Pinto, A.; Rai, R.; Monteiro, M.; Carvalho, P.; Mendonça, P.; Peleteiro, M.C.; Parra, F.; Duarte, M.D. Detection of Recombinant Hare Myxoma Virus in Wild Rabbits (Oryctolagus cuniculus algirus). Viruses 2020, 12, 1–12, doi:10.3390/v12101127
90%	Q1 I.F. 5.005 5-year I.F. 4.761	**Abade dos Santos, F.A**.; Carvalho, C.L.; Monteiro, M.; Carvalho, P.; Mendonça, P.; Peleteiro, M.C.; Duarte, M.D. Recombinant myxoma virus infection associated with high mortality in rabbit farming (Oryctolagus cuniculus). Transbound. Emerg. Dis. 2020, 1–6, doi:10.1111/tbed.13899.

90%	Q1 I.F. 5.005 5-year I.F. 4.761	**Abade dos Santos**, F.A., Dalton, K.P. Carvalho, C.L., Casero, M., Álvarez, A.L., Parra, F., Duarte, M.D. Co-infection by classic MYXV and ha-MYXV in Iberian hare (Lepus granatensis) and Wild rabbit (Oryctolagus cuniculus algirus). *Transboundary and emerging diseases*, 10.1111/tbed.14540. Advance online publication.
90%	Q1 I.F. 4.422 5-year I.F. 5.513	**Abade dos Santos, F.A**., Carvalho, C.L., Valente P.C.L.G, Armés H. ,Reemers S. S., Peleteiro M.C., Sanz, I.C., Dalton K.P., Parra F., Duarte M.D. Evaluation of commercial myxomatosis vaccines against recombinant myxoma virus (ha-MYXV) in Iberian hare and wild rabbit. Vaccines, 10(3), 356. https://doi.org/10.3390/vaccines10030356
80%	Q1 I.F. 3.340 5-year I.F. 3.272	**Abade dos Santos, F.A**.; Monteiro, M.; Pinto, A.; Carvalho, C.L.; Peleteiro, M.C.; Carvalho, P.; Mendonça, P.; Carvalho, T.; Duarte, M.D. First description of a herpesvirus infection in genus Lepus. *PLoS One* **2020**, *15*, e0231795–e0231795, doi:10.1371/journal.pone.0231795.