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Early circulation of rabbit haemorrhagic disease virus type 2 in domestic and wild lagomorphs in southern California, USA (2020–2021)

Javier Asin[1] | Daniel Rejmanek[2] | Deana L. Clifford[3] | Andrea B. Mikolon[4] | Eileen E. Henderson[1] | Akinyi C. Nyaoke[1] | Melissa Macías-Rioseco[5] | Nicolas Streitenberger[1] | Juliann Beingesser[1] | Leslie W. Woods[2] | Antonio Lavazza[6] | Lorenzo Capucci[6] | Beate Crossley[2] | Francisco A. Uzal[1]

[1] California Animal Health and Food Safety Laboratory System, San Bernardino branch, University of California-Davis, San Bernardino, California, USA

[2] California Animal Health and Food Safety Laboratory System, Davis branch, University of California-Davis, Davis, California, USA

[3] Wildlife Health Laboratory, California Department of Fish and Wildlife, Rancho Cordova, California, USA

[4] California Department of Food and Agriculture, Sacramento, California, USA

[5] California Animal Health and Food Safety Laboratory System, Tulare branch, University of California-Davis, Tulare, California, USA

[6] Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna and OIE Reference Laboratory for Rabbit Hemorrhagic Disease, Brescia, Italy

Correspondence
Javier Asin, California Animal Health and Food Safety Laboratory System, San Bernardino branch, University of California-Davis, San Bernardino, CA 92408, USA.
Email: jasinros@ucdavis.edu

Javier Asin and Daniel Rejmanek contributed equally.

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Abstract

Rabbit haemorrhagic disease virus type 2 (RHDV2) causes a severe systemic disease with hepatic necrosis. Differently from classic RHDV, which affects only European rabbits (*Oryctolagus cuniculus*), RHDV2 can affect many leporid species, including hares (*Lepus* spp.) and cottontail rabbits (*Sylvilagus* spp.). RHDV2 emerged in Europe in 2010 and spread worldwide. During the last 5 years, there have been multiple outbreaks in North America since the first known event in 2016 in Quebec, Canada, including several detections in British Columbia, Canada, between 2018 and 2019, Washington State and Ohio, USA, in 2018 and 2019, and New York, USA, in 2020. However, the most widespread outbreak commenced in March 2020 in the southwestern USA and Mexico. In California, RHDV2 spread widely across several southern counties between 2020 and 2021, and the aim of this study was to report and characterize these early events of viral incursion and circulation within the state. Domestic and wild lagomorphs ($n = 81$) collected between August 2020 and February 2021 in California with a suspicion of RHDV2 infection were tested by reverse transcription quantitative real-time PCR on the liver, and histology and immunohistochemistry for pan-lagovirus were performed on liver sections. In addition, whole genome sequencing from 12 cases was performed. During this period, 33/81 lagomorphs including 24/59 domestic rabbits (*O. cuniculus*), 3/16 desert cottontail rabbits (*Sylvilagus audubonii*), and 6/6 black-tailed jackrabbits (*Lepus californicus*) tested positive. All RHDV2-positive animals had hepatic necrosis typical of pathogenic lagovirus infection, and the antigen was detected in sections from individuals of the three species. The 12 California sequences were closely related (98.9%–99.95%) to each other, and also very similar (99.0%–99.4%) to sequences obtained in other southwestern states during the 2020–2021 outbreak; however, they were less similar to strains obtained in New York in 2020 (96.7%–96.9%) and Quebec in 2016 (92.4%–92.6%), suggesting that those events could be related to different viral incursions. The California sequences were more similar (98.6%–98.7%)

to a strain collected in British Columbia in 2018, which suggests that that event could have been related to the 2020 outbreak in the southwestern USA.

KEYWORDS
California, lagomorphs, rabbit, RHD, RHDV2

1 | INTRODUCTION

Classic rabbit haemorrhagic disease (RHD) is caused by rabbit haemorrhagic disease virus (RHDV), a single-stranded, positive-sense RNA virus that, together with the European brown hare syndrome virus (EBHSV), belongs to the family *Caliciviridae*, genus *Lagovirus* (Capucci et al., 2021; ICTV, 2019; OIE, 2021). The *Lagovirus* genus includes other non-pathogenic viruses, overall named rabbit caliciviruses (RCVs) or hare caliciviruses (HCVs). Due to the frequent recombination events observed among circulating lagoviruses and the increasing number of reported sequences, a new taxonomy based on phylogenetic relationships has been proposed to classify the *Lagovirus* genus into distinct genogroups and genotypes, e.g., GI.1, GI.2, etc (Le Pendu et al., 2017). The RHDV genome is ~7.4 kb and is divided into two open reading frames (ORFs). ORF1 encodes a polyprotein that splits into several non-structural proteins and the major structural capsid protein, VP60. ORF2 encodes a minor structural protein called VP10 (Abrantes et al., 2012; Ohlinger et al., 1990; Wirblich et al., 1996). Classic RHD has an incubation period of 1–3 days, a very rapid clinical course, and high mortality (70%–90%). It affects only animals older than 6–8 weeks. Despite rare detections in stored samples from Iberian hares (*Lepus granatensis*) (Lopes et al., 2014), classic RHDV is believed to affect only domestic and wild European rabbits (*Oryctolagus cuniculus*) (Lavazza et al., 1996; OIE, 2021). Clinical signs include bleeding through nares and other body orifices, lethargy, and seizures, but frequently there is only sudden death (Abrantes et al., 2012). The main lesion is severe hepatic necrosis (Marcato et al., 1991). Since its first detection in China in 1984, RHDV spread to most of the world. In the USA, there have been punctual detections of RHDV and a close relative, the Michigan rabbit calicivirus, believed to be a non-pathogenic RCV that attempted a gain in pathogenicity (Abrantes et al., 2012; Bergin et al., 2009; Campagnolo et al., 2003); however, a pathogenic lagovirus never became endemic in the country.

In 2010, a genetically and antigenically distinct virus named rabbit haemorrhagic disease virus type 2 (RHDV2; or GI.2 according to Le Pendu et al., 2017) emerged in France (Capucci et al., 2021; Le Gall-Reculé et al., 2011, 2013). RHDV2 constitutes a phylogenetically different genotype having more than 15% divergence with other lagoviruses, including RHDV and the non-pathogenic RCVs and HCVs (Dalton et al., 2015). Despite these differences, the organization of the RHDV2 genome is similar to RHDV and other caliciviruses (Dalton et al., 2015; Wirblich et al., 1996). This new virus also affects kits younger than 6–8 weeks (Dalton et al., 2012; Le Gall-Reculé et al., 2013) and has a much broader host range than classic RHDV, including multiple hare and jackrabbit species (*Lepus* spp.) and cottontail rabbits (*Sylvilagus* spp.), in addition to wild and domestic European rabbits (Asin et al., 2021; Lankton et al., 2021; Le Gall-Reculé et al., 2017; Neimanis, Ahola, et al., 2018). The incubation period may range between 3 and 9 days, and some reports describe a slightly longer disease course than with classic RHD, which might result in higher numbers of subacutely to chronically affected individuals (Abade Dos Santos et al., 2020; Le Gall-Reculé et al., 2013), but, overall, both viruses produce similar lesions (Calvete et al., 2018; Neimanis, Ahola, et al., 2018). Mortality associated with RHDV2 infection varies across different reports; it was initially considered lower than with classic RHDV, but there have been recent descriptions of more virulent RHDV2 strains with similar mortality, incubation period, and disease course as classic RHDV (Capucci et al., 2017; OIE, 2021). This new virus rapidly spread across Europe and the rest of the world and, as of July 2021, it has been detected in several European countries, Australia, Africa, North America, and Asia (Ambagala, Ababio, et al., 2021; Fukui et al., 2021; Hu et al., 2021; Rouco et al., 2019).

In North America, there have been several detections of RHDV2 since the first report in 2016 in Quebec, Canada (Ambagala, Schwantje, et al., 2021; Rouco et al., 2019; USDA-APHIS, 2020). In 2018, a phylogenetically different RHDV2 strain caused high mortality in colonies of feral domestic rabbits on the coast of British Columbia, Canada (USDA-APHIS, 2018). There were further detections in Vancouver (British Columbia), Canada, in 2019, but the viral sequences differed from those obtained the previous year in the same region (Ambagala, Schwantje, et al., 2021; USDA-APHIS, 2018, 2020). At the same time, RHDV2 was detected in feral and domestic European rabbits in Washington State and Ohio, USA, in 2018 and 2019 (USDA-APHIS, 2020; Williams et al., 2021). Sequences from the Quebec 2016 episode differed from those detected in British Columbia in 2018 and 2019 (USDA-APHIS, 2020), and so far, no strain similar to the first known event in Quebec has been detected in North America. In March 2020, the virus killed several domestic rabbits in a clinic of New York City, USA; the detected strains differed from those collected in British Columbia in 2018 and were more similar to the strains detected in the same area of Canada the following year (Ambagala, Schwantje, et al., 2021; USDA-APHIS, 2020). Since its first detection in New Mexico, USA, on 24 March 2020, an outbreak of RHDV2 spread through wild and domestic leporid populations across the entire southwestern USA, Mexico, and, most recently, some northern states of the USA and southern Canada (Government of Alberta, 2021; USDA-APHIS, 2020, 2021). To date, RHDV2 is considered endemic in a vast area of the southwestern USA, and it has been detected in the following states as part of

this current outbreak: New Mexico, Arizona, Texas, Colorado, Nevada, California, Utah, Wyoming, Montana, Oregon, Idaho, South Dakota, Georgia, and Florida (USDA-APHIS, 2021). In California, the virus was detected for the first time on 07 May 2020, in a wild black-tailed jackrabbit (*Lepus californicus*) that was found dead close to Palm Springs (Riverside County; southern California) (Asin et al., 2021). The disease was detected in domestic rabbits soon after, with the first confirmed case on 10 July 2020 in a backyard rabbitry of the San Bernardino County. To date, the outbreak has affected several counties in the state, including Riverside, San Bernardino, Los Angeles, San Diego, Orange, Kern, Ventura, Alameda, and San Luis Obispo counties (CDFA, 2021; USDA-APHIS, 2021).

Whole genome sequencing (WGS) of RHDV2 strains from Arizona, New Mexico, and Texas obtained between March and April 2020 revealed a unique genetic cluster that differentiates those strains from viral sequences obtained in New York during March of the same year (O'Donnell et al., 2021). During prior emergence and incursion of RHDV2 in other continents, WGS of the circulating stains has been performed to establish viral origin and evolution (Abrantes et al., 2020; Buehler et al., 2020; Lopes et al., 2019; Mahar et al., 2018; Silvério et al., 2018). In addition, sequencing of isolates from domestic and wild species in a given geographical area may demonstrate clustering by high nucleotide homology, thus supporting spillover between sympatric individuals (Velarde et al., 2017). To date, viral sequences from California have not been published.

In California, other states of the USA, and other American countries, there are numerous wild lagomorphs, including several hare and jackrabbit species, multiple cottontail rabbit species, pygmy rabbits (*Brachylagus idahoensis*), American pikas (*Ochotona princeps*), and volcano rabbits (*Romerolagus diazi*) (Chapman & Flux, 2008; Jameson & Peeters, 2004). Some of these species are not naturally present in other parts of the world, thus the introduction of RHDV2 in the American continent is exposing new wild lagomorph species to the virus and broadening its host range (Asin et al., 2021; Lankton et al., 2021; O'Donnell et al., 2021). In contrast to the situation in some countries of southern Europe, there is no widespread industrial production of rabbit meat in California and the USA in general, since it is not as popular among consumers (Lukefahr et al., 2004). Instead, many operations consist of backyard rabbitries with small numbers of animals that are bred for mixed purposes, such as exhibition or personal meat consumption. Rabbits are also bred and used for biotechnology purposes (i.e. medical/pharmaceutical industry) or to serve as food for companion animals and captive wildlife. Lastly, rabbits are very popular pets and owners frequently have more than one individual at home (AVMA, 2018).

In this study, we present data derived from the first months of the RHDV2 outbreak in California as part of the general epizootic that is occurring in the USA since March 2020 (USDA-APHIS, 2020, 2021). Our data include whole genome sequences obtained from RHDV2-positive domestic and wild leporid species, some of them affected for the first time in this outbreak. In addition, microscopic pathology and antigen detection in liver sections of domestic rabbits and newly affected wild species are presented.

2 | MATERIALS AND METHODS

2.1 | Animals tested and case histories

A total of 81 wild and domestic leporids with a suspicion of RHD were submitted to the California Animal Health and Food Safety Laboratory system (CAHFS; University of California-Davis) laboratories between 09 August 2020 and 25 February 2021. These included 59 domestic rabbits, 16 cottontail rabbits, and six black-tailed jackrabbits. Within the domestic rabbit category, there were 18 rabbits from backyard rabbitries, 30 rabbits that were kept as pets, 10 feral domestic rabbits, and one laboratory rabbit. Within the cottontail rabbit category, there were nine desert cottontails (*Sylvilagus audubonii*), three cottontails of not specified species (*Sylvilagus* spp.), and four Riparian brush rabbits (*Sylvilagus bachmani riparius*). Liver samples ($n = 81$) were tested by reverse transcription quantitative real-time PCR (RT-qPCR; see below). Clinical histories provided by the submitters of the wild species were recorded. In addition, owners of the domestic rabbits that tested positive were provided a standardized set of questions to get the same baseline information from each episode. Questions included (1) breed; (2) purpose of the animal/group (pet/backyard rabbitry); (3) number of animals in premises; (4) number of animals lost; (5) clinical signs noted; (6) location of the animals (indoor/outdoor); (7) duration of the episode (days between the death of the first rabbit and the death of the last rabbit); (8) vaccination status at the moment of the episode and/or willingness to vaccinate in the future (i.e. to the survivors or if more rabbits are acquired).

2.2 | Postmortem examinations, histopathology, and immunohistochemistry

Necropsies were performed on all animals submitted. Fresh liver tissue samples were collected and used for molecular analyses as described below. Liver from each case was also fixed in 10% neutral buffered formalin for 24–48 h, embedded in paraffin, processed routinely for the production of 5-μm sections, stained with haematoxylin and eosin, and evaluated for the presence of necrosis typical of pathogenic lagovirus infection by light microscopy (Marcato et al., 1991). Selected liver sections (three from domestic rabbits, three from cottontail rabbits, and three from black-tailed jackrabbits) were immunostained with a primary anti-lagovirus antibody cocktail (6G2, 3H6, and 6D6; mouse monoclonal, concentration 1 mg/ml; obtained from the OIE Reference Laboratory for RHD, Brescia, Italy) that targets different capsid epitopes of pathogenic and non-pathogenic lagoviruses (Capucci et al., 1995). The antibody was used at a concentration of 1:700 following a previously described protocol (Neimanis, Ahola, et al., 2018). Liver sections

of RHD-unaffected animals of the three species were used as negative controls.

2.3 | Sample preparation and RNA extraction

Fresh liver tissue samples were homogenized using a MagNA Lyser (Roche Diagnostics). Briefly, 250 mg of tissue was placed in a 2 ml polypropylene tube containing 1.5 ml denaturation solution (Ambion #8540G) and filled a quarter full with silica beads. The sample was homogenized at 6500 rpm for 45 s and then incubated at room temperature for 5 min. Next, 20 μl of Proteinase K (20 mg/ml) was mixed with 20 μl of homogenate and incubated at 56°C for 60 min. The digest was then transferred to a MagMax 96 Express plate (Thermo Fisher) with 20 μl of binding beads and 100 μl lysis solution per sample. The plate was transferred to a BioSprint magnetic processor (Qiagen), and RNA extraction was performed using the AM1836 extraction protocol as described in the MagMax-96 Viral RNA Isolation Kit user guide (Thermo Fisher).

2.4 | TaqMan RT-qPCR

A one-step multiplex RT-qPCR kit (TaqMan Fast Virus 1-Step; Thermo Fisher), designed to target the *vp60* gene, was used to amplify RHDV2 viral RNA (Duarte et al., 2015). Each PCR reaction was set up in a 25 μl volume containing 6.25 μl of buffer, 7.25 μl nuclease-free water, 2.5 μl of each primer, 0.5 μl of probe, 1 μl Xeno internal control, and 5.0 μl of RNA template or controls. A RHDV2-positive liver sample, verified by the United States Department of Agriculture (USDA), was used as a positive control. Nuclease-free water was used as a negative control. The thermocycling reactions were performed using a 7500 Fast PCR System (Applied Biosystems) under the following conditions: reverse transcription at 50°C for 5 min, reverse transcriptase inactivation/initial denaturation at 95°C for 20 s, and 45 cycles of amplification and extension (95°C for 3 s and 60°C for 30 s). Cycle threshold values below 35 were considered positive.

2.5 | WGS and phylogenetic analyses

Extracted total RNA was used as template for double-stranded cDNA synthesis using the Maxima H Minus kit (Thermo Scientific). Thirteen μl of template RNA and 1 μl of random hexamers were used in the synthesis reaction following the manufacturer's instructions. The resulting double-stranded cDNA was purified using AMPure XP beads (Beckman Coulter) and used as input to a library generated with the Ligation Sequencing Kit SQK-LSK109 [Oxford Nanopore Technologies (ONT)]. First, DNA was end-repaired using the NEBNext Ultra II DNA End Prep and Repair kit (New England Biolabs), purified using AMPure XP beads in a ratio of 1:1 volume of beads per sample and eluted in 30 μl nuclease-free water. Sequencing adapters (AMX) (ONT) were ligated to the DNA using NEBNext Quick T4 DNA ligase (New England Biolabs) by incubation at room temperature for 10 min. The adapter-ligated DNA library was purified with AMPure XP beads in a ratio of 1:2.5 volume of beads per sample, followed by two washes with S Fragment buffer (ONT) and elution in 7 μl elution buffer (ONT). The library was loaded onto a Flongle Flow Cell R9.4.1 (ONT) and run via MinKNOW software for 24 h. Base-called FASTQ files containing 'pass' reads (Q-score ≥7) were loaded into Geneious Prime and mapped to a reference RHDV2 genome (GenBank: MT506237) using the Minimap2 plugin. Consensus sequences with a minimum coverage of at least 50X across the entire genome were submitted to GenBank and given the following accession numbers: MW926371 – MW926381 and MW926383).

A multiple sequence alignment of the RHDV2 genomes was assembled in Geneious Prime using the MUSCLE alignment algorithm. In addition to the 12 genomes sequenced in the current study, the following sequences and associated GenBank accession numbers were included in the alignment: RHDV2 Arizona 2020 (MT506237), RHDV2 Australia 2016 (MF421696), RHDV2 Canada 2016 (KY235675), RHDV2 Canada 2018 (MT900570), RHDV2 Canada 2019 (MT900574), RHDV2 Germany 2017 (MN901451), RHDV2 Netherlands 2016 (MN061492), RHDV2 New Mexico 2020 (MT506234), RHDV2 New York 1 2020 (MT506236), RHDV2 New York 2 2020 (MT506235), RHDV2 Poland 2018 (MN853661), RHDV2 Texas 2020 (MT506233). Phylogenetic analysis using a Maximum Likelihood method was performed with the programme MEGA (Version 6). The Tamura 3-parameter model of nucleotide substitution with a discrete Gamma distribution was used. Bootstrap values were calculated using 1000 pseudo-replicates.

TABLE 1 Animals tested by rabbit haemorrhagic disease virus type 2 (RHDV2) RT-qPCR on liver tissue in California between August 2020 and February 2021

	Positive	Negative	Total
Domestic	24	35	59
Cottontail	3	13	16
Jackrabbit	6	0	6
Total	33	48	81

Domestic, domestic rabbit (*O. cuniculus*); cottontail, cottontail rabbit species not specified and desert cottontail rabbit (*Sylvilagus* spp. and *Sylvilagus audubonii*); jackrabbit, black-tailed jackrabbit (*L. californicus*).

3 | RESULTS

3.1 | RT-qPCR results and case histories

A total of 33/81 (40.7%) animals tested positive by RT-qPCR from the liver in this period (Table 1). Details about the 33 RHDV2-positive animals can be found in Table 2. Among the positive individuals of wild species ($n = 9$), 3/9 had a history of simply 'found dead'. In 4/9 individuals, the submitter reported blood around body orifices (most commonly

TABLE 2 Data of rabbit haemorrhagic disease virus type 2 (RHDV2)-positive cases collected in California between August 2020 and February 2021

Accession number[§]	Species	Collection date	County	Age	Sex	Ct (liver)	GenBank accession
S2101453	Domestic[†]	16/02/2021	RIV	A	M	13.67	-
D2101776	Jackrabbit	08/02/2021	KC	U	U	11.82	-
S2101296	Jackrabbit	04/02/2021	SD	A	U	11.51	MW926381
S2100812	Domestic[†]	26/01/2021	LA	A	F	13.14	-
S2100668	Domestic[†]	17/01/2021	RIV	A	M	10.92	-
S2100495A	Domestic[‡]	13/01/2021	LA	A	M	12.19	
S2100495B	Domestic[‡]	13/01/2021	LA	K	U	10.7	-
S2100208	Domestic[‡]	07/01/2021	RIV	A	F	11.8	MW926380
S2100071	Domestic[†]	05/01/2021	SBD	A	F	13.55	-
S2100006A	Domestic[†]	01/01/2021	RIV	K	M	13.03	-
S2100006B	Domestic[†]	01/01/2021	RIV	A	M	13.01	-
S2100006C	Domestic[†]	01/01/2021	RIV	A	U	13.67	-
S2100006D	Domestic[†]	01/01/2021	RIV	A	U	13.59	-
S2010381	Domestic[‡]	23/12/2020	RIV	A	F	12.07	-
T2002842	Domestic[‡]	18/12/2020	KC	A	M	13.8	MW926383
T2002819	Jackrabbit	14/12/2020	KC	A	F	11.49	-
D2014863	Domestic[†]	09/12/2020	KC	A	F	14.14	-
D2015091	Jackrabbit	04/12/2020	KC	U	M	10.08	-
T2002721A	Domestic[‡]	03/12/2020	KC	A	U	10.02	-
T2002721B	Domestic[‡]	03/12/2020	KC	K	U	10.72	-
S2009752	Jackrabbit	02/12/2020	LA	A	M	11.10	MW926379
D2100068	Cottontail	02/12/2020	LA	A	U	11.99	MW926374
D2014271	Jackrabbit	28/11/2020	KC	U	F	11.93	MW926373
D2014044	Cottontail	21/11/2020	KC	U	U	11.06	MW926371
D2014080	Cottontail	10/11/2020	KC	U	U	10.58	MW926372
S2009110	Domestic[‡]	09/11/2020	LA	A	M	12.48	MW926378
S2008982	Domestic[‡]	04/11/2020	RIV	A	F	12.43	MW926377
S2007194A	Domestic[†]	03/09/2020	SBD	A	F	12.68	-
S2007194B	Domestic[†]	03/09/2020	SBD	K	M	13.33	MW926376
S2007194C	Domestic[†]	03/09/2020	SBD	K	M	13.15	-
S2006894A	Domestic[†]	24/08/2020	SBD	A	F	11.74	MW926375
S2006894B	Domestic[†]	24/08/2020	SBD	A	F	12.24	-
S2006894C	Domestic[†]	24/08/2020	SBD	A	M	12.4	-

A, adult (≥6 months); K, kit (<6 months); M, male; F, female; U, unknown; RIV, Riverside; SD, San Diego; KC, Kern; LA, Los Angeles; SBD, San Bernardino; Ct, cycle threshold value in liver RT-qPCR (Ct <35 is considered as positive).
Domestic, domestic rabbit (*O. cuniculus*); cottontail, desert cottontail rabbit (*S. audubonii*); jackrabbit, black-tailed jackrabbit (*L. californicus*).
[†]Domestic rabbit from a backyard rabbitry.
[‡]Domestic rabbit kept as a pet.
[§]Same accession number with different letter indicates that individual rabbits belong to the same owner/premises.

nares, but also anus in one case). Carcasses of 5/9 wild animals were found in areas where other dead cottontails or jackrabbits had been seen recently. The 24 RHDV2-positive domestic rabbits belonged to 15 different premises/households, and 14/15 of those owners answered the standardized questions. In 6/14 premises, rabbits were kept as pets; 8/14 premises were backyard rabbitries with 14 to 55 animals (median: 24) and different purposes, which included breeding for exhibition, to provide breeder stock supply to other owners, to be sold as pets, for meat production for private consumption, and, most commonly, for a combination of several of these purposes. There were rabbits of multiple breeds without any particular predominant breed, and in 6/14 premises there were animals of several different breeds mixed.

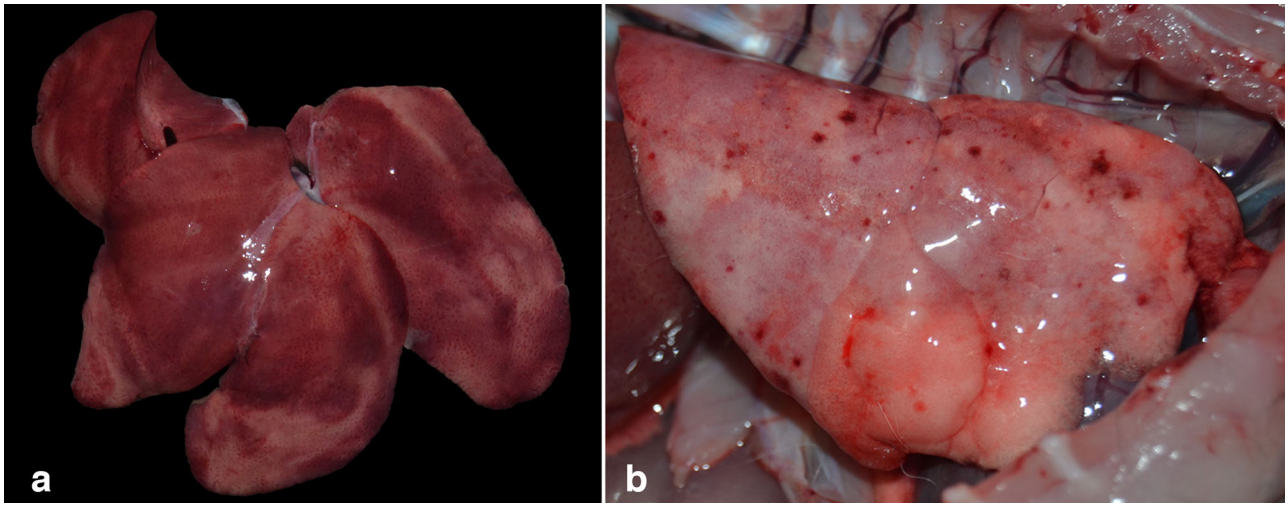

FIGURE 1 Gross findings in a rabbit haemorrhagic disease virus type 2 (RHDV2)-positive domestic rabbit (*O. cuniculus*). (a) Liver. Hepatomegaly with alternating areas of superficial reddening and pallor, and diffusely accentuated lobular/reticular pattern. (b) Lung. Multiple petechial haemorrhages on the surface.

In 6/14 premises, animals were always kept indoors, whereas in 8/14 they were outdoors. Clinical signs noted by the owners varied from none (i.e. just 'found dead') to brief episodes of lethargy and/or anorexia prior to death; 4/14 owners reported blood coming out of body orifices (nares and/or anus) in some of the rabbits. Mortality ranged from 11.4% to 100% (median: 48.1%), and the duration of the episode on the premises was 3–22 days (median: 3.5). None of the rabbits was vaccinated at the time of the episode; 3/14 owners had already vaccinated the survivors when the questions were asked, 9/14 were willing to have their rabbits vaccinated in the future, 1/14 was not willing to vaccinate, and 1/14 had lost all the rabbits and was not getting any more. Vaccine hesitancy arguments included an elevated price of each individual dose (especially among owners of multiple rabbits), limited availability in some geographical locations, and difficulty in moving groups of rabbits to vaccination spots.

3.2 | Necropsy findings, liver histopathology, and lagovirus antigen detection

Gross necropsy findings in RHDV2-positive animals were similar to previously described cases of RHD (Asin et al., 2021; Lankton et al., 2021; Neimanis, Ahola, et al., 2018) (Figure 1). Histologically, all RHDV2-positive animals had periportal to panlobular hepatic necrosis typical of pathogenic lagovirus infection (Marcato et al., 1991) (Figure 2). Sections from some individuals had moderate to severe freezing and thawing or postmortem decomposition artifact, but groups of necrotic hepatocytes could still be detected. Lagovirus antigen was detected in association with the areas of necrosis in all the immunostained liver sections from RHDV2-positive animals (Figure 2), which included some poorly preserved sections. No hepatic necrosis or lagovirus antigen was detected in any of the negative controls.

3.3 | WGS and phylogenetic analyses

All 12 viral genomes were successfully sequenced in their entirety. The strains were all closely related, sharing between 98.9% and 99.95% nucleotide identity (Figure 3a). There was a degree of geographical clustering by high nucleotide homology (99.6%–99.95%) among some of the sequences such as D2014044/cottontail and D2014271/jackrabbit or D2100068/cottontail, S2009752/jackrabbit, and S2009110/domestic (Figure 3b). Other sequences such as T2002842/domestic and S2007194B/domestic also shared a high degree of nucleotide identity (99.7%) despite being geographically separated, whereas others were more dissimilar despite being geographically closer, as in the case of S2008982/domestic and S2100208/domestic (99.4% nucleotide identity). The 12 California strains were also closely related to several recently characterized strains from Arizona, New Mexico, and Texas (O'Donnell et al., 2021), sharing between 99.0% and 99.4% nucleotide identity. However, the California strains formed their own, unique, highly supported branch on the phylogenetic tree.

Additionally, as evidenced by the phylogenetic grouping, the California strains were less similar to the New York 2020 strains (96.7%–96.9% identity) and even more dissimilar (92.4%–92.6% nucleotide identity) to a strain collected in 2016 in Quebec (Canada 2016) during the first known event of incursion of RHDV2 in North America. The California sequences were more similar to a strain obtained in 2018 in British Columbia (Canada 2018), with a nucleotide identity of 98.6%–98.7%. However, they were dissimilar to a strain collected in the same area the following year (Canada 2019; 96.9%–97.0% nucleotide identity) that clustered with the mentioned New York 2020 strains. On the intercontinental level, the 12 California sequences shared between 92.1% and 92.8% nucleotide identity with European strains collected in the Netherlands, Germany, and Poland between 2016 and 2018, and

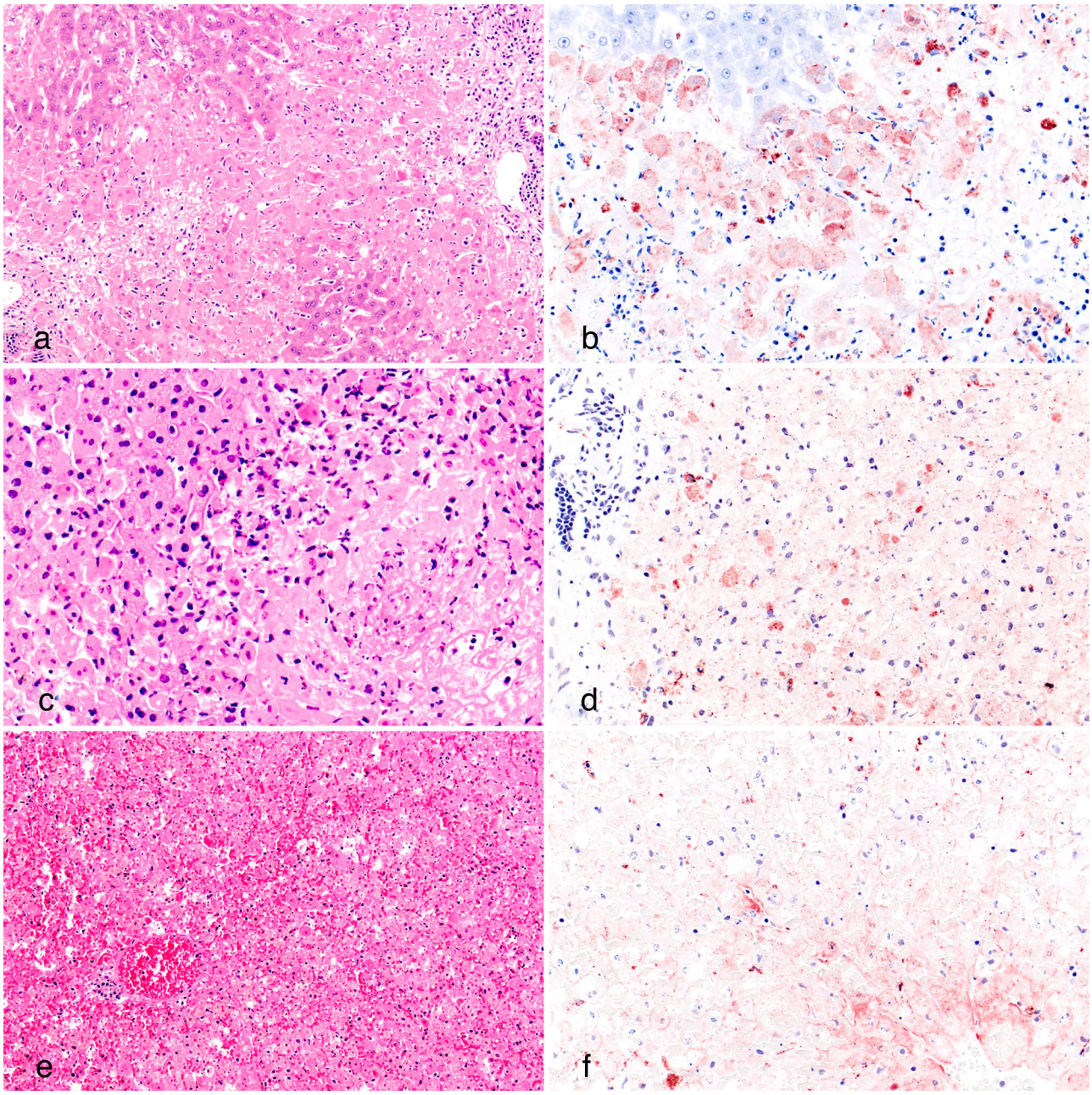

FIGURE 2 Hepatocellular necrosis (a, c, and e; haematoxylin and eosin) and positive antigen detection (b, d, and f; pan-lagovirus immunohistochemistry) in the liver of rabbit haemorrhagic disease virus type 2 (RHDV2)-positive domestic and wild leporids from California. (a and b) Domestic rabbit (*O. cuniculus*). (c and d) Desert cottontail (*S. audubonii*). (e and f) Black-tailed jackrabbit (*L. californicus*)

were more dissimilar (87.6%–87.8%) to a strain collected in Australia in 2016.

Recent phylogenetic analyses of European RHDV2 strains have identified a number of recombinant viruses (Abrantes et al., 2020; Buehler et al., 2020; Lopes et al., 2019; Silvério et al., 2018). To test whether any of the California strains showed evidence of recent recombination, we created two additional phylogenetic trees representing the non-structural genes (nucleotides 1–5294) and structural genes (nucleotides 5295–7375). The topology of each tree was nearly identical to that of the whole genome tree (data not shown), indicating no evidence of recent recombination events.

4 | DISCUSSION

In this study, we present data from the first months of the 2020–2021 RHDV2 outbreak in California, including whole genome sequences obtained from 12 wild and domestic leporids in which lagovirus-associated hepatic lesions and presence of viral antigen were demonstrated.

Some of the RHDV2-positive wild animals were found dead in areas where other carcasses had been seen in the previous days, suggesting undetected virus circulation in certain geographical locations, as described in other parts of the world (Mahar et al., 2018;

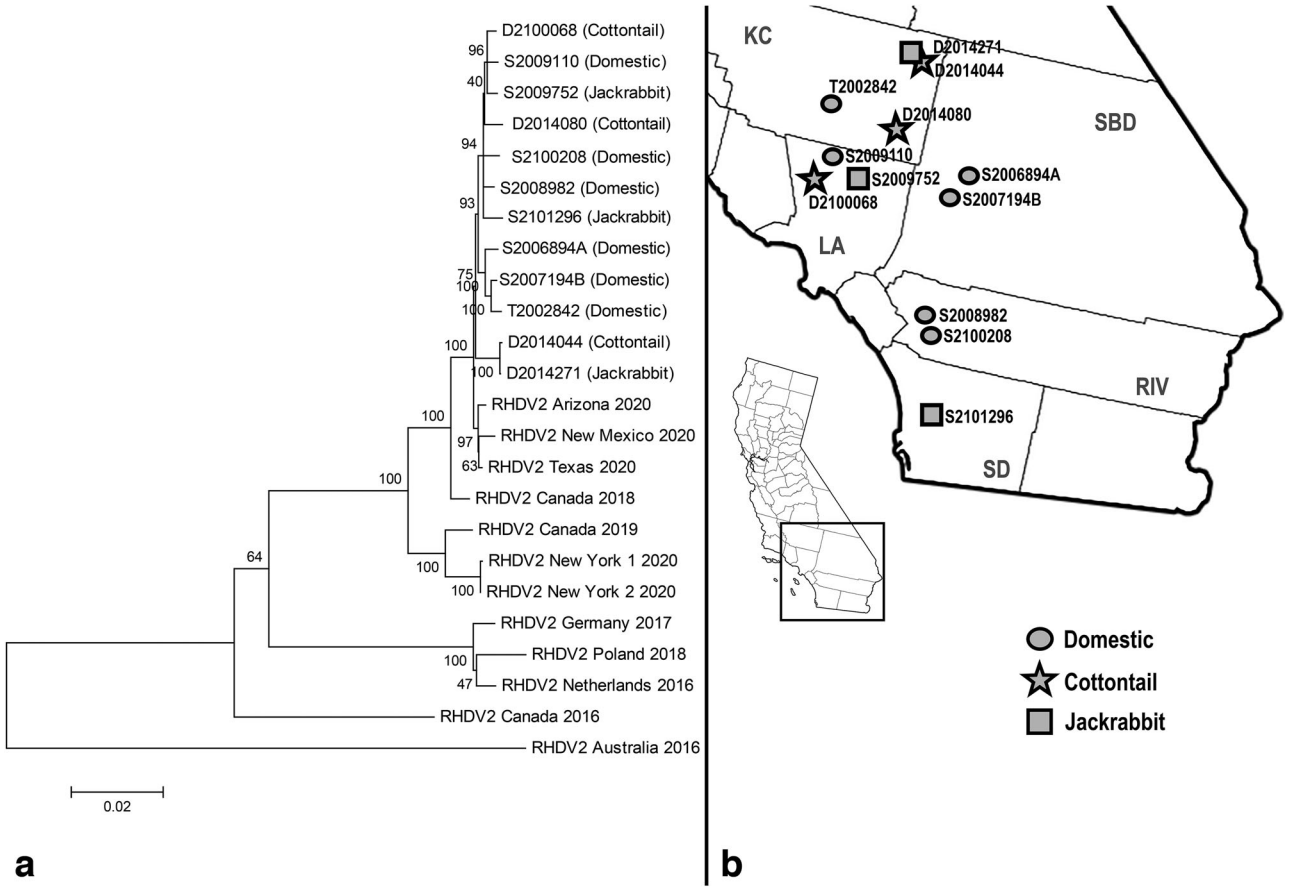

FIGURE 3 (a) Maximum likelihood phylogenetic tree using whole genome rabbit haemorrhagic disease virus type 2 (RHDV2) sequences from the California outbreak (leporid species in parenthesis) collected between August 2020 and February 2021, recent strains collected in the southwestern United States in 2020, several European strains collected between 2016 and 2018, and a 2016 strain from Australia. Horizontal branch lengths are drawn to scale of nucleotide substitutions per site and the percentage of trees in which the associated taxa clustered together was determined from 1000 bootstrap replicates and is shown at each node. GenBank accession numbers: RHDV2 Arizona 2020 (MT506237), RHDV2 Australia 2016 (MF421696), RHDV2 Canada 2016 (KY235675), RHDV2 Canada 2018 (MT900570), RHDV2 Canada 2019 (MT900574), RHDV2 Germany 2017 (MN901451), RHDV2 Netherlands 2016 (MN061492), RHDV2 New Mexico 2020 (MT506234), RHDV2 New York 1 2020 (MT506236), RHDV2 New York 2 2020 (MT506235), RHDV2 Poland 2018 (MN853661), RHDV2 Texas 2020 (MT506233), S2101296 (MW926381), S2100208 (MW926380), T2002842 (MW926383), S2009752 (MW926379), D2100068 (MW926374), D2014271 (MW926373), D2014044 (MW926371), D2014080 (MW926372), S2009110 (MW926378), S2008982 (MW926377), S2007194B (MW926376), and S2006894A (MW926375). (b) Geographical location of cases sequenced in the period in California. KC, Kern county; SBD, San Bernardino county; LA, Los Angeles county; RIV, Riverside county; SD, San Diego county; domestic, domestic rabbit (*O. cuniculus*); cottontail, desert cottontail (*S. audubonii*); jackrabbit, black-tailed jackrabbit (*L. californicus*)

Neimanis, Ahola, et al., 2018). RHDV2 circulation among wild species is a threat for domestic populations, and the most probable explanation for disease emergence in domestic rabbits in California just 4 months after the first detection in a black-tailed jackrabbit (Asin et al., 2021). In fact, backyard rabbitries are very popular in certain parts of the state, although it is difficult to estimate the exact number, which complicates monitoring efforts. In our set, we report outbreaks not only in groups of domestic rabbits that were kept outdoors, but also in animals that were exclusively indoors, possibly associated with the action of mechanical vectors and fomites in disease transmission (Asgari et al., 1998). Sanguineous nasal discharge is considered a classic clinical sign of this disease (Marcato et al., 1991; OIE, 2021); however, only four out of 14 owners of domestic rabbits reported this finding, suggesting that the absence of this sign does not necessarily preclude a presumptive diagnosis of RHD, which is in agreement with the results of a large-scale study carried out in Spanish commercial farms (Rosell et al., 2019). Viral spread in our backyard settings was fast, ranging between 3 and 4 days in most cases, and the mortality (11.4%–100%) was similar to that of the more virulent RHDV2 strains detected from 2014 to 2015 (Capucci et al., 2017; OIE, 2021).

Hepatic necrosis is a hallmark of pathogenic lagovirus infection (Abrantes et al., 2012; Calvete et al., 2018; Marcato et al., 1991; Neimanis, Ahola, et al., 2018), and all 33 positive animals included in this study had this microscopic lesion. Interestingly, and as recently suggested (Lankton et al., 2021), RHDV2 seems to induce similar hepatic lesions in the individuals of the native leporid species included in this study, and lagoviral antigen was detected in the areas of necrosis of these animals as previously reported in domestic rabbits and hares

(Neimanis, Larsson, et al., 2018; Neimanis, Ahola, et al., 2018). Indeed, RHDV2 had spilled over to multiple hare species (Camarda et al., 2014; Hall et al., 2017; Puggioni et al., 2013; Velarde et al., 2017), thus its detection in black-tailed jackrabbits was somehow expected, and just adds another susceptible species within the *Lepus* genus. Detection in rabbits of the genus *Sylvilagus* is rather new (Lankton et al., 2021), and our study shows intralesional lagoviral antigen in a species of this genus, the desert cottontail, for the first time. In this line, up to 20% of an invading population of a close relative, the eastern cottontail (*Sylvilagus floridanus*), in northern Italy had detectable antibodies against EBHSV, which suggests a degree of susceptibility in rabbits of this genus to infection by certain pathogenic lagoviruses (Lavazza et al., 2015). To our knowledge, there were only two descriptions of an RHD-like disease in cottontails in the scientific literature before this outbreak. Lavazza et al. (2015) detected an EBHSV-infected eastern cottontail during an EBHS outbreak in Italy, and subsequently reproduced the disease in one out of four eastern cottontails inoculated with EBHSV, but not in those inoculated with classic RHDV. A recent study performed at Plum Island Animal Disease Center (New York, USA) with eastern cottontails demonstrated that this species is susceptible to RHDV2, since three out of five experimentally infected individuals died with typical lesions and both viral RNA and antigen were detectable in several tissues (Mohamed et al., 2018). Our data are still limited, but suggest that desert cottontails develop disease when they are naturally infected with RHDV2; however, a certain degree of resistance in animals of the genus *Sylvilagus* cannot be totally excluded.

Our 12 California strains are very similar to those collected in wild and domestic lagomorphs in other southwestern states during 2020 (O'Donnell et al., 2021), therefore suggesting a single event of viral introduction as the origin of this outbreak. The California strains, however, already formed a well-differentiated branch in the phylogenetic tree despite originating from the first months of dissemination within the state, which points to a well-established viral circulation in the area (Mahar et al., 2018). In addition, there was a degree of clustering among some California strains, including high nucleotide identity between viruses obtained from sympatric wild and domestic species (e.g. in Los Angeles County), which suggests interspecies transmission. In fact, spillover between a broad range of lagomorph species is an important characteristic of RHDV2 (Le Gall-Reculé et al., 2017; Velarde et al., 2017), and this study confirms that the virus is behaving similarly in North America. Nevertheless, there were also some California strains that were highly similar despite not being geographically close, which possibly indicates other means of viral dissemination different to sympatric transmission between species. Some of these means may have involved transport over longer distances by scavenger birds, insects, or even human intervention (e.g. via movement of rabbits, use of contaminated hay or other products, etc), especially in the case of domestic species (e.g. cases T2002842 and S2007194B). In this line, human intervention is considered a key factor in the rapid, worldwide spread of RHDV2 (Rouco et al., 2019).

The southwestern sequences are dissimilar to strains obtained in the Canada 2016 and New York 2020 outbreaks (Ambagala, Schwantje, et al., 2021; USDA-APHIS, 2020). As suggested by O'Donnell et al. (2021), this points to different, possibly concomitant events of viral incursion in North America in the past 5 years. Nevertheless, a sequence obtained in British Columbia in 2018 (Canada 2018) (Ambagala, Schwantje, et al., 2021) partially clusters with the southwestern USA strains, including our 12 California strains. Interestingly, this strain is more similar to the California strains (98.6%–98.7% nucleotide identity), than to Canada 2016 (92.7%) and New York 2020 (97.3%), which suggests that the southwestern USA outbreak may have originated from a strain that was introduced into British Columbia in 2018. In fact, between 2018 and 2019, there were RHDV2 detections in Washington State and Ohio (USDA-APHIS, 2020; Williams et al., 2021) and, although WGS data are not available in GenBank for confirmation yet, a recent notice by the USDA stated that these sequences are highly homologous to those detected in British Columbia in 2018 (USDA-APHIS, 2020), which would support this hypothesis. Interestingly, a strain collected in British Columbia in 2019 (Canada 2019) differed from the strains obtained the previous year in the same area and was more closely related to the New York 2020 strains. This may suggest two independent events of viral incursion into western Canada, from which the virus was then subsequently introduced into different areas of the USA (Ambagala, Schwantje, et al., 2021).

The North American strains, including our 12 California sequences, are more similar to European than to Australian strains collected from 2016 onwards. In fact, RHDV2 originated in France in 2010 (Le Gall-Reculé et al., 2011), and in 10 years has spread to five different continents (Ambagala, Ababio, et al., 2021; Fukui et al., 2021; Hu et al., 2021; Rouco et al., 2019). WGS and *vp60* sequencing data from different parts of the world have confirmed that the circulating strains derive from European viruses (Mahar et al., 2018; O'Donnell et al., 2021). Our analysis thus reinforces that the different events of viral introduction in North America may have had a common European ancestor. To date, events of RHDV2 introduction from Australia to North America are unlikely to have occurred. Abrantes et al. (2020) demonstrated that the Canada 2016 and Netherlands 2016 strains were recombinants between a non-pathogenic GI.3 strain (RCV-E1), which was the donor for the non-structural part of the genome, and a strain of the novel pathogenic RHDV2 (GI.2), which donated the structural part. The phylogenetic tree topologies of the structural and non-structural segments in our analysis, which contains both the Canada 2016 and Netherlands 2016 strains, are identical, therefore it may be deduced that our California strains are likely GI.3/GI.2 recombinants as well. There is no evidence of more recent recombination, which is consistent with the recent introduction of this virus into North America.

RHDV2 has the potential to inflict profound alterations in new ecosystems where it is introduced (Delibes-Mateos et al., 2014; Monterroso et al., 2016). Lessons from prior RHDV2 epizootics in other parts of the world should be applied to California and the USA in general. For instance, in certain areas of the Iberian Peninsula such as Aragón (northern Spain), a decline in wild rabbit (*O. cuniculus*) populations was reported in 2013, coinciding with the emergence of RHDV2 (Delibes-Mateos et al., 2014). This event paralleled with a similar decreasing trend in the numbers of Iberian lynx (*Lynx pardinus*) and Spanish imperial eagle (*Aquila adalberti*), which are both endangered

predators that rely heavily on a rabbit-based diet (Delibes-Mateos et al., 2014; Monterroso et al., 2016). In California, there are established populations of wild felids such as mountain lions (*Puma concolor*) and bobcats (*Lynx rufus*), coyotes (*Canis latrans*), and golden eagles (*Aquila chrysaetos*) (CDFW, 2021; Roberts & Crimmins, 2010), for which rabbit is also an important component of the diet, thus the current RHDV2 dissemination might also negatively affect those native predators. In addition, there are five lagomorph species of conservation concern, including, but not limited to, the state and federally endangered Riparian brush rabbit (CNDDB, 2021).

Due to the close interaction between wild and domestic lagomorphs, its broader host range, and the evolution of similar epizootics in other continents, it is reasonable to think that RHDV2 has come to California to stay. Our study includes whole genomic sequences of strains that have circulated during the first months of the outbreak and should serve as a cornerstone for further studies and monitoring efforts in different events of viral incursion in North America and other parts of the world.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

ETHICS STATEMENT

The authors confirm that the ethical policies of the journal, as indicated in the journal's author guidelines website, have been adhered to. No ethical approval was required as examined animals were part of the routine diagnostic activities of CAHFS labs in collaboration with CDFA and CDFW.

DATA AVAILABILITY STATEMENT

Data supporting the findings of this article are available in the corresponding database (GenBank) and/or upon request from the corresponding author.

ORCID

Javier Asin https://orcid.org/0000-0002-6178-4801
Antonio Lavazza https://orcid.org/0000-0002-7646-3592
Lorenzo Capucci https://orcid.org/0000-0002-1830-3929
Francisco A. Uzal https://orcid.org/0000-0003-0681-1878

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