Incursions of rabbit hemorrhagic disease virus 2 in Canada – clinical, molecular and epidemiological investigation

Aruna Ambagala[1,*], Helen Schwantje[2], Sonja Laurendeau[3], Heindrich Snyman[4], Tomy Joseph[4], Bradley Pickering[1], Kathleen Hooper-McGrevy[1], Shawn Babiuk[1], Estella Moffat[1], Lindsey Lamboo[1], Oliver Lung[1], Melissa Goolia[1], Mathieu Pinette[1], Carissa Embury-Hyatt[1]

[1]National Centre for Foreign Animal Disease, Canadian Food Inspection Agency, Winnipeg, MB. [2]British Columbia Ministry of Forests, Lands, Natural Resource Operations and Rural Development, Nanaimo, BC. [3]Animal Health - Traceability and Terrestrial Animal Disease Control, Montreal QC. [4]Animal Health Centre, Ministry of Agriculture, Abbotsford, BC.

* Corresponding author

[#] Current address: Animal Health Laboratory, University of Guelph, Kemptville, Ontario, Canada

Abstract:

Rabbit hemorrhagic disease virus 2 (RHDV2) is a newly emerging *Lagovirus* belonging to the family *Caliciviridae*. After its first discovery in 2010 in France, this highly pathogenic virus rapidly spread to neighboring countries and has become the dominant strain, replacing the classical RHDV1 strains. RHDV2 was first reported in North America in 2016 in Mont-Joli, Quebec, Canada and it was reported again in 2018 and 2019 on Vancouver, Island and the southwest mainland of British Columbia (BC). The whole genome sequence of the RHDV2 Quebec isolate resembled the RHDV-N11 isolate from Navarra, Spain identified in 2011 with 97% identity. The epidemiological investigation involved three hobby farms and one personal residence. In December and February 2018, high mortality was reported in first a private feral rabbit refuge and then, a large colony of feral rabbits on the Vancouver Island University Campus, Nanaimo, BC. The virus responsible showed only 93% identity to the Quebec RHDV2 isolate at the nucleotide level. Additional cases of RHDV2 on Vancouver Island and on the BC mainland affecting feral, captive domestic and commercial rabbits were reported subsequently. Vaccination was recommended to control the outbreak and an inactivated bivalent vaccine was made available to the private veterinary practices. In June 2019 an isolated RHDV2 outbreak was reported in an apartment building in Vancouver, BC. This virus showed only 97% identity to the RHDV2 isolate responsible for the BC outbreak in 2018 at the nucleotide level suggesting that it was an independent incursion. In October 2020, there are reports of partial recovery of the feral population in Nanaimo and to date there are no confirmed deaths of native rabbit species in BC.

Introduction

Rabbit hemorrhagic disease (RHD) is a highly contagious viral hepatitis that affects wild and domesticated European rabbits. It was first reported in China in 1984, and has since spread globally (Liu et al., 1984; Park et al., 1987; Gregg and House, 1989; Cancellotti et al., 1991; Embury-Hyatt et al., 2012). The clinical signs include loss of appetite, lethargy, pyrexia, muscle fasciculations, bleeding from the nose, moth and anus and sudden death. In per-acute cases rabbits die of RHD without any clinical signs (Abrantes et al., 2012). The causative agent, RHD virus (RHDV) is a non-enveloped, positive-sense, single-stranded RNA virus belonging to the Family *Caliciviridae.* Historically, based on the sequence of the structural capsid protein gene *vp60*, this virus was divided into six genogroups G1–G6 (Le Gall-Reculé et al., 2003). In 2010, a new RHDV variant RHDV2 emerged in France (Le Gall-Reculé et al., 2011), and unlike the 'classical' RHDV (RHDV1), RHDV2 was pathogenic in rabbits of all age groups, and affected a number of Mediterranean, European and Alpine hare species including *Lepus capensis*, *Lepus corsicanus*, *Lepus europaeus* and *Lepus timidus* (Camarda et al., 2014; Hall et al., 2017; Le Gall-Reculé et al., 2017; Puggioni et al., 2010; Velarde et al., 2017). RHDV2 is currently endemic in Europe, and outbreaks have been recently reported from Canada, USA, China, Ireland, Netherlands, UK, Tunisia, Morocco, Senegal, Côte d'Ivoire and Ghana (Bel et al., 2019; Dalton et al., 2015; Fitzner and Niedbalski, 2018; Hu et al., 2020; Miao et al., 2019; Neimanis et al., 2018; Rocchi et al., 2019; WAHIS OIE Reports). Rabbits vaccinated against the classical RHDV1 are not completely protected against RHDV2 strains (Bárcena et al., 2015, Müller et al., 2019).

The first case of RHD in Canada was reported in 2011 in a one year-old male rabbit from Winnipeg, Manitoba that died of acute liver failure (Embury-Hyatt et al., 2012). Nucleic acid

63 sequencing of the *vp60* gene of the virus revealed a 98% identity to an RHDV1 isolate from
64 China (GenBank accession number DQ530363.1). An epidemiological investigation of the index
65 premise revealed that the affected rabbit was purchased from a local pet store in the spring of
66 2010. Two additional rabbits living at the same premises and sharing the same litter box with the
67 dead rabbit did not develop any disease, and they tested negative for RHDV nucleic acid and
68 antibodies to RHDV. Despite an extensive epidemiological investigation, no potential source of
69 infection was identified.

70 **RHDV2 in Quebec**

71 On August 17th, 2016, the Canadian Food Inspection Agency (CFIA) received a call from the
72 Ministère de l'Agriculture, des Pêcheries et de l'Alimentation du Québec (MAPAQ) Animal
73 Pathology Laboratory in Québec city reporting a suspicion of RHD in a dead 4-month-old male
74 rabbit from a hobby farm in Mont-Joli. On necropsy, the rabbit was in good body condition,the
75 trachea contained some pinkish fluid, the left lung was markedly congested and the liver was
76 enlarged with rounded edges and accentuated lobular architecture. The kidneys were dark red,
77 and the urinary bladder contained slightly pink urine. Liver, spleen, and kidney samples from the
78 rabbit were submitted to the National Centre for Foreign Animal Disease (NCFAD), Winnipeg
79 (NCFAD Lab # WIN-AH-2016-OTH-0018). Ten percent homogenates in sterile phosphate
80 buffered saline (PBS) supplemented with antibiotics were made from each tissue and subjected
81 to phase contrast electron microscopy and RHDV-specific conventional reverse transcription
82 PCR (RT-PCR) as described previously (Embury-Hyatt et al., 2012). Under the electron
83 microscope ~30 nm diameter calicivirus-like particles were observed (Figure 1A), and all three
84 tissue samples tested positive for RHDV genomic material by the conventional RT-PCR (Figure
85 1B). The identity of the amplicons were confirmed RHDV2 by Sanger Sequencing.

For pathotyping of the RHDV, three, 8-week old New Zealand White rabbits were inoculated with the 10% liver homogenate (Embury-Hyatt et al., 2012). The next day morning, all animals appeared healthy, however a few hours later one rabbit was found dead, another displayed neurological signs (circling), and the third one was reluctant to move. Five hours later, the remaining two rabbits were found dead. On post-mortem, no significant gross lesions were observed likely because the disease was per-acute. However, microscopic lesions were observed, and were similar to those seen in RHVD2 BC (NCFAD Lab # WIN-AH-2018-OTH-0024) infected rabbits as described below. Total nucleic acid extracted from a liver sample from one of the rabbits was subjected to next-generation sequencing on an Illumina MiSeq instrument. BLAST analysis showed that the whole genome sequence of the RHDV2 recovered (GenBank Accession # KY235675) had 97% identity to the RHDV-N11 isolate (GenBank Accession # KM878681) at the nucleotide level. The RHDV-N11 isolate obtained from dead young rabbits (28 days old) from Navarra, Spain in 2011, infected both adult rabbits and neonates (Alda et al., 2010), and did not agglutinate human RBC type O as do the previously known RHDV1 strains. The RHDV2 QC whole genome sequence showed only 84.47% nucleotide identity to the 2011 MB RHDV1 isolate (GenBank Accession # KY235676).

On August 19th, 2016, the hobby farm in Mont-Joli (Premise A) was quarantined, movement restriction was imposed and an epidemiological investigation was initiated by the CFIA. The investigation revealed that the Premise received 5 rabbits from a hobby farm located in Ste-Flavie, Québec (Premise B). A few weeks after the 5 rabbits were introduced, 13 out of 18 rabbits in the farm died (72% mortality). During the time of investigation, the Premise A reported that several weeks before two rabbits developed apathy, anorexia, pyrexia, jaundice and seizures. They were treated by a private veterinarian with antibiotics for Pasteurellosis and both

recovered. The two recovered rabbits were euthanized by the CFIA on August 22nd 2016 and blood, serum and fresh tissues (liver, kidney and lungs) were submitted to the NCFAD (Lab #WIN-AH-2016-OTH-0021). The blood and tissue samples were negative for RHDV genomic material by conventional RT-PCR, but the serum samples were positive for antibodies to RHDV by competitive ELISA (RHDV serological kit, OIE Reference Laboratory for RHD, Istituto Zooprofilattico Sperimentale della Lombardia e dell, 'Emilia Romagna, Italy) confirming that those two animals had been exposed to RHDV. Premise B had also experienced high mortality (89%) after the 5 rabbits were sold to Premise A. At the time of the investigation, only one rabbit remained alive at Premise B as a free-roaming rabbit. It was captured, euthanized and samples (blood, serum, fresh tissues) were submitted to the NCFAD. The blood sample and tissues were negative by RHDV-specific conventional RT-PCR, however sera was positive for antibodies against to RHDV by ELISA (NCFAD Lab # WIN-AH-2016-OTH-0023). Prior to the high mortality observed in Premise B, 3 rabbits were bought from Premise C and one rabbit from Premise D**.**

Premise C was a hobby farm located in Ste-Angèle de Mérici, Quebec, it had sold 3 rabbits at the end of July 2016, and 5 rabbits in mid-May 2016 to Premise B. Premise C also sold one rabbit to Premise A at the end of July. At the time of investigation a total of 7 rabbits were present on-site. All rabbits appeared healthy and there was no history of high mortality indicative of RHD in the farm. The rabbits were sampled (blood, serum, feces) on three consecutive 5 day interval (August 24th, 29th and on October 4th) and tested negative for RHDV genetic material and antibodies to RHDV at the NCFAD (Lab # WIN-AH-2016-OTH-0022,-0025,-0029).

Premise D was a private residence, and when the CFIA investigated the premise on August 24th, 2016 no rabbits were present on-site. The owner had 2 breeder rabbits obtained in September

2015 from a regional source. A litter of 4 rabbits was born in February 2016 and 3 of them died in March 2016. The 2 breeder rabbits died in early April 2016 and the sole juvenile survivor was sold to Premise B around the 2nd or 3rd week of July 2016. Based on this epidemiological investigation it was concluded that Premise D appeared to be the source of the outbreak. Cleaning and disinfection of all four premises were approved by CFIA on October 14th and quarantine was imposed for 60-days. In order to mitigate the risk of spread, CFIA contacted a local veterinary clinic on August 22nd to validate the cleaning and disinfection protocol and to initiate tracing activities on all potentially exposed rabbits having gone through the clinic since August 9th. The epidemiological investigation revealed no evidence of feed, bedding (both purchased from local sources) or any travel linked to the outbreak.

RHDV2 in BC

In December 2017, an outbreak affecting a feral (unowned European domestic rabbits (*Oryctolagus cuniculus*)) colony of rabbits on private property south of Nanaimo, BC was reported. The property owner held "100s" of rabbits in a refuge but did not provide additional details or samples but claimed almost the entire population had died. In February 2018, high mortality (approximately 30 rabbits over two weeks) was observed in a large population of feral rabbits (European origin) on the Vancouver Island-University Campus in Nanaimo. Students were concerned for malicious poisoning so on February 14th, three of seven dead rabbits found dead on in the University Campus were submitted to the Animal Health Centre (AHC) in Abbottsford, BC. All three rabbits were in good body condition with adequate muscle mass, fat reserves and normal hydration status. Post-mortem revealed no obvious gross lesions except for mild diffuse pulmonary edema and congestion in one of the rabbits. No sign of foreign material

155 or easily recognizable toxic compounds were observed in the stomach content and tissues were
156 submitted for histopathology, bacteriology (for both aerobic and anaerobic cultures) and for
157 toxicology (carbamate, organoposphates and anticoagulant rodenticides). No toxins were found
158 in the samples and the bacteriology and PCR results were negative for *Salmonella*, *Francisella*
159 *tularensis*, and *Clostridium pilliforme*. Histopathology revealed marked widespread acute
160 hepatocellular necrosis, disseminated lymphocyte karyorrhexis and necrosis in the spleen white
161 pulp, presence of extensive intra-glomerular capillary thrombosis, and scattered renal tubular
162 necrosis in all three animals. Since the histopathological lesions were highly suggestive of RHD,
163 pooled visceral organ samples were tested by RHDV-specific conventional nested PCR at the
164 AHC. All samples tested positive for RHDV and immediately sent to the NCFAD for
165 confirmation. Additional necropsies were performed on March 3, 2018 at the Wildlife Health
166 facility in Nanaimo and all rabbits showed gross pathological changes consistent with RHD;
167 ecchymotic hemorrhages of uterine serosa, lungs, epicardium and swollen, pale livers with
168 accentuated lobular architecture. At the NCFAD (Lab # WIN-AH-2018-OTH-0024) samples
169 were confirmed positive for RHDV2 by conventional PCR followed by Sanger sequencing.
170 Presence of live virus in the samples and pathogenesis was confirmed by inoculating four 8-
171 weeks old New Zealand White rabbits with clarified 10% tissue suspension from one of the liver
172 samples submitted. One rabbit was found dead the next day and three others were euthanized due
173 to severe depression and inability to stand. Liver and spleen tissue collected from all four rabbits
174 were positive for RHDV2. At post-mortem examination, enlarged pale livers with enhanced
175 zonal pattern reflecting hepatocellular necrosis was observed in all four rabbits (Fig. 2A and 2B),
176 and in one animal a fissure in the liver was observed (Fig. 2B). Hemorrhages were observed in
177 both the lungs (Fig. 2C) and heart (Fig. 2D) in two of the animals. By histopathology, periportal

178 to mid-zonal hepatocellular necrosis with multifocal periportal non-suppurative hepatitis was
179 observed (Fig 3A). In situ-hybridization was performed using an RHDV-*vp60* specific RNA
180 probe V-RHDV-pp-O2 (Cat#. 485781) synthesized by ACD, Newark, CA. RHDV RNA could
181 be detected extensively within hepatocytes in the periportal regions (Fig.3B). In the kidney,
182 extensive micro-thrombosis of glomerular capillaries was observed (Fig. 3C) and RHDV RNA
183 was detected primarily within glomeruli and associated glomerular arterioles and interstitial
184 blood vessels (Fig. 3D). The splenic red pulp was expanded by the presence of eosinophilic
185 fibrillar material consistent with fibrin and there were scattered degenerating cells with pyknotic
186 or fragmented nuclei (Fig. 3E). Within the white pulp there were areas of hemorrhage and
187 lymphocytolysis. Abundant amounts of RHDV RNA were observed by *in-situ* hybridization
188 throughout the spleen (Fig. 3F). In the lungs, there were large areas of hemorrhage/edema,
189 multifocal perivascular accumulations of heterophils and numerous vascular micro-thrombi (Fig.
190 3G). Abundant viral RNA could be detected in the lung primarily within the alveolar septa
191 (Fig.3H). In addition to the epicardial hemorrhages observed grossly, there were multifocal
192 hemorrhages within the myocardium and occasional micro-thrombi were observed (Fig. 3I).
193 Viral RNA was primarily detected in association with the capillaries in the intercellular spaces
194 (Fig. 3J). Viral RNA was also detected in association with blood vessels in the thymus (not
195 shown). Total nucleic acid extracted from liver from one of the four rabbits were subjected to
196 next generation sequencing and whole genome sequence of RHDV2 was obtained (GenBank
197 Accession # MT900570). At that time, BLAST analysis identified the RHDV-N11 from Navarra,
198 Spain as the closet match (93.23%) confirming that this virus was different from that was
199 responsible for the 2016 RHDV2 outbreak in Quebec. The RHDV2 2018 BC isolate showed
200 only 92.74% identity to the RHDV2-QC-2016 isolate at the whole genome level.

On March 21, 2018 NCFAD received an additional pool of spleen and kidney from four dead rabbits from the AHC, for RHDV confirmation (Lab # WIN-AH-2018-OTH-0029). The tissues were from a submission from a colony of 9 feral rabbits that died on February 13th, 2018 on Annacis Island in Delta, BC. These dead rabbits were initially identified by a local resident and were bought to the Delta Animal Shelter where they were frozen on February 14th, 2018. Media reports of the detection of RHDV2 on Vancouver island had prompted the subsequent submission of 4 of these frozen rabbits to the AHC on March 14th, 2018. Gross post mortem changes consisted of mild diffuse pulmonary edema and congestion, diffuse liver pallor and subtle accentuation of the hepatic lobular architecture. Pooled visceral organ samples from each rabbit tested positive through the RHDV-specific conventional nested PCR at the AHC. RHDV2 was subsequently confirmed by the NCFAD in all tissues by conventional PCR and Sanger sequencing and total nucleic acid extracted from all four pooled liver samples were subjected to next generation sequencing. One of the RHDV-2 whole genome sequences was identical to the Nanaimo BC RHDV-2 whole genome sequence, and the other two showed 4 (GenBank Accession # MT900571) and 11 single nucleotide polymorphisms.

On April 09, 2018 NCFAD received frozen liver, spleen and kidney samples from a dead rabbit suspected to be of Eastern cottontail rabbit *(Sylvilagus floridanus)* origin (WIN-AH-2018-OTH-0029). The rabbit was found on March 21 2018 in a field in Courtenay, BC less than 500 m from where numerous domestic feral rabbits were found dead. All the samples were positive for RHDV by conventional PCR and nucleic acid extracted from the liver sample was subjected to next generation sequencing. The whole genome sequence (GenBank Accession # MT900572) was identical to that of the two previous BC RHDV2 whole genome sequences (GenBank Accession # MT900570 and MT900571). Genetic testing of organ DNA extracts at the

Agriculture and Food Laboratory - University of Guelph later confirmed that the rabbit was in fact a feral European rabbit and not an Eastern cottontail.

In April 2018, the inactivated RHD vaccines Filavac VHD K C+V (Filavie, Roussay – France) which contains both killed RHDV1 and RHDV2 viruses and was imported into BC and made available to the private veterinary practices in BC. Additional cases of RHDV were reported until the end of June 2018 on the BC mainland (Delta, and Richmond areas) and on Vancouver Island (Errington, Coombs, Parksville, Courtenay and Comox) involving feral, domestic rabbits and commercial rabbitries.

On March 31, 2019, four feral rabbits at Trillium Lodge in Parksville on Vancouver Island were found dead and submitted to the AHC. Tissues from 3 of the 4 dead rabbits tested positive by conventional PCR at the AHC. The samples were sent to NCFAD (Lab # WIN-AH-2019-OTH-0022) for confirmation and genomic sequencing. The samples were confirmed positive for RHDV2 by conventional PCR and Sanger sequencing. Whole genome sequence (GenBank Accession # MT900573) showed 99.5% sequence identity to the RHDV-2-BC-2018 whole genome sequences.

On June 14, 2019, AHC, BC received two Havana rabbits that died unexpectedly a day before. The rabbits were from a group of 10 rabbits raised in poor sanitary conditions in an apartment building in downtown Vancouver, BC. The dead rabbits were frozen and both gross pathology and histopathological evaluation were inconclusive. Tissue samples from both rabbits tested positive for RHDV at the AHC and samples were submitted to the NCFAD (Lab # WIN-AH-2019-0032) for confirmation and whole genome sequencing. The whole genome sequencing results identified a RHDV2 virus (GenBank Accession # MT900574) with only 97% similarity

to the previously isolated RHDV2 viruses in BC indicating that this virus was not related to the 2018 RHDV2 outbreak. The Province's Chief Veterinary Officer issued a general order under the BC Animal Health Act to stop owners from moving rabbits in or out of the apartment building and mandated vaccination for RHD in that building.

Conclusion

RHDV2 strains are rapidly spreading and replacing the classical RHDV strains globally (Rouco et al., 2019). The outbreak in Mont-Joli, Quebec in 2016 was the first reported RHDV2 outbreak in the Americas. This outbreak was rapidly contained and controlled without vaccination. The 2018 BC outbreak in feral rabbits was a new incursion and not related to the Quebec outbreak. The outbreak involved feral rabbits of European origin and later spread to privately owned rabbits and rabbitries on Vancouver Island and on the southwest BC mainland. In order to control the spread, vaccination of domestic rabbits was implemented. The outbreak subsided by the end of summer 2018 but remerged in 2019 on a small scale. The outbreak killed a large number of feral rabbits in this specific area of BC, however there were no reports of involvement of diseased local non-European rabbits or hare species. Vancouver Island has no native rabbit or hare species but does have an abundance of Eastern cottontail rabbits as well as feral rabbit populations. The BC mainland does have a number of native rabbit species including snowshoe hares (*Lepus americanus*), white-tailed jackrabbits (*Lepus townsendii*) and Nuttall's cottontails (*Sylvilagus nuttallii*), but these occur regionally and may simply not have been in contact with the sources of RHD. Eastern cottontails (*Sylvilagus floridanus*) from an introduction to BC in the 1920's are also present in areas of the southeast mainland.

Starting in July 2019, RHDV-2 outbreaks were reported in Washington State, USA, mainly in domestic rabbits of European origin. Additional cases of RHDV2 in domestic rabbits were also reported in 2020 in a number of US states including New York, Nevada, Colorado, New Mexico and Texas. In addition to the rabbits of European origin, black-tailed jackrabbits (*Lepus californicus*) in Colorado, and desert cottontail rabbits (*Sylvilagus audubonii*) in Nevada and California (OIE, WAHIS) were affected in those outbreaks. The RHDV2 strains responsible for the recent outbreaks in Texas, Arizona and New Mexico are closely related to the RHDV2 strain responsible for the BC 2018 feral rabbit outbreak both at both VP60 and whole genome level. The RHDV strain that was responsible for the death of Havana rabbits in Vancouver, BC in 2019 was genetically more similar to the RHDV2 strains isolated from New York (Figure 4a and 4b). Although many whole genome sequences of RHDV have been added to the GenBank recently, the whole genome sequence of the RHDV2 2016 isolate from Quebec remains closely related to the to the RHDV2 2011 isolate from Navarra, Spain (Figure 4b). However at the VP60 sequence level (Figure 4a), it now more closely matches the VP60 sequences of RHDV2 strains isolated during 2015-2018 period in Tunisia (Rahli et al., 2019).

In all three independent RHDV2 incursions in Canada, no source of introduction was found. During 2018-2019 BC outbreak tracing was difficult due to the widespread nature and pattern of mortality in feral, unowned animals. In the 2015 Quebec outbreak, considering that clinical signs suggestive of RHD were observed as early as mid/end of July in 2016 on hobby farm (B) and that the surviving rabbit had antibodies to the RHDV, it is reasonable to assume that hobby farm (B), via the sale of 5 rabbits on August 1st to hobby farm (A), was the source of contamination for hobby farm (A). The source of infection for hobby farm (B) could be exposure of the rabbits to a contaminated environment via the free movements of cats and dogs, or roaming coyotes

observed on site. Another possible mode of introduction could be via the sale of an asymptomatic carrier from the personal residence (D) around the 2nd or 3rd week of July. Considering that rabbits were no longer present on the site of the personal residence (D), this hypothesis could not be validated.

Acknowledgements

Dr Caroline Dubé, DMV, Ph. D., CFIA Epidemiologist and Scientific Advisor, Animal Health Risk Assessment Section and Dr Julie Paré, DMV, Ph. D., CFIA Surveillance Epidemiologist, Terrestrial Animal Health Epidemiology & Surveillance Section for the data analysis; The following CFIA Québec Area staff for the data collection: Sophie Benoit, B.Sc., technical specialist, Dr Yves Bélanger, DMV, District Veterinarian, Dr Hélène Massé, DMV, Animal Health Veterinarian and the Animal Feed, Plant Protection Division. We also like to thank Dr. Valerie Smid and the Animal Staff at the NCFAD for the animal experiments, Lynn Burton and Andre Dufresne for electron microscopy and Cody Buchanan for whole genome sequencing.. We thank veterinary pathologists and laboratory staff from the Animal Health Center, BC Ministry of Agriculture for their help with the initial detection of RHDV in BC.

Conflict of Interest

None of the authors of this paper has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

Data Availability statement

All data related to this manuscript will be made available upon request from the corresponding author

References:

1. Abrantes J, van der Loo W, Le Pendu J, Esteves PJ. Rabbit haemorrhagic disease (RHD) and rabbit haemorrhagic disease virus (RHDV): a review. Vet Res. 2012 Feb 10;43(1):12.
2. Alda F, Gaitero T, Suárez M, Merchán T, Rocha G, Doadrio I (2010) Evolutionary history and molecular epidemiology of rabbit haemorrhagic disease virus in the Iberian Peninsula and Western Europe. BMC Evol Biol 10(10):347. doi:10.1186/1471-2148-10-347
3. Bell DJ, Davis JP, Gardner M, et al. Rabbit haemorrhagic disease virus type 2 in hares in England. Vet Rec. 2019;184(4):127-128.
4. Bárcena J, Guerra B, Angulo I, González J, Valcárcel F, Mata CP, Castón JR, Blanco E, Alejo A. Comparative analysis of rabbit hemorrhagic disease virus (RHDV) and new RHDV2 virus antigenicity, using specific virus-like particles. Vet Res. 2015 Sep 24;46(1):106.
5. Camarda A, Pugliese N, Cavadini P, Circella E, Capucci L, Caroli A, Legretto M, Mallia E, Lavazza A. 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. 2014;97(3):642–5.
6. Cancellotti FM, Renzi M. 1991. Epidemiology and current situation of viral haemorrhagic disease of rabbits and the European brown hare syndrome in Italy. Rev Sci Tech 10:409–422.CAL

7. Dalton KP, Abrantes J, Lopes AM, et al. Complete genome sequence of two rabbit hemorrhagic disease virus variant b isolates detected on the Iberian Peninsula. Arch Virol. 2015;160(3):877-881.
8. Embury-Hyatt C, Postey R, Hisanaga T, et al. The first reported case of rabbit hemorrhagic disease in Canada. Can Vet J. 2012;53(9):998-1002.
9. Fitzner A, Niedbalski W. Detection of rabbit haemorrhagic disease virus 2 (GI.2) in Poland. Pol J Vet Sci. 2018;21(3):451-458.
10. Gregg DA, House C. 1989. Necrotic hepatitis of rabbits in Mexico: a parvovirus. Vet Rec 125:603–604.CAL
11. Hall RN, Peacock DE, Kovaliski J, Mahar JE, Mourant R, Piper M, Strive T. Detection of RHDV2 in European brown hares (*Lepus europaeus*) in Australia. Vet Rec. 2017 Feb 4;180(5):121.
12. Hu B, Wei H, Fan Z, et al. Emergence of rabbit haemorrhagic disease virus 2 in China in 2020. Vet Med Sci. 2020;10.
13. Le Gall-Reculé G, Lemaitre E, Bertagnoli S, Hubert C, Top S, Decors A, Marchandeau S, Guitton J-S (2017) Large-scale lagovirus disease outbreaks in European brown hares (*Lepus europaeus*) in France caused by RHDV2 strains spatially shared with rabbits (*Oryctolagus cuniculus*). Vet Res 48 (1):70.
14. Le Gall-Reculé G, Zwingelstein F, Boucher S, Le Normand B, Plassiart G, Portejoie Y, Decors A, Bertagnoli S, Guérin JL, Marchandeau S. Detection of a new variant of rabbit haemorrhagic disease virus in France. Vet Rec. 2011 Feb 5;168(5):137-8.
15. Le Gall-Reculé G, Zwingelstein F, Laurent S, de Boisséson C, Portejoie Y, Rasschaert D. Phylogenetic analysis of rabbit haemorrhagic disease virus in France between 1993 and

2000, and the characterization of RHDV antigenic variants. Arch Virol. 2003 Jan;148(1):65-81.

16. Liu SJ, Xue HP, Pu BQ, Qian NH. 1984. A new viral disease in rabbits. Anim Husbandry Vet Med 16:253–255.

17. Miao Q, Qi R, Veldkamp L, et al. Immunogenicity in Rabbits of Virus-Like Particles from a Contemporary Rabbit Haemorrhagic Disease Virus Type 2 (GI.2/RHDV2/b) Isolated in The Netherlands. Viruses. 2019;11(6):553.

18. Müller C, Ulrich R, Schinköthe J, Müller M, Köllner B. Characterization of protective humoral and cellular immune responses against RHDV2 induced by a new vaccine based on recombinant baculovirus. Vaccine. 2019 Jul 9;37(30):4195-4203.

19. Neimanis AS, Ahola H, Zohari S, et al. 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. 2018;65(1):213-220.

20. OIE Report Event summary: Rabbit hemorrhagic disease, United States of America - WAHIS Last updated 02/07/2020

21. Puggioni G, Cavadini P, Maestrale C, Scivoli R, Botti G, Ligios C, Le Gall-Reculé G, Lavazza A, Capucci L. The new French 2010 rabbit hemorrhagic disease virus causes an RHD-like disease in the Sardinian cape hare (*Lepus capensis mediterraneus*). Vet Res. 2013;44.

22. Park NY, Chong CJ, Kim JH, Cho SM, Cha YH, Jung BT, Kim DS, Yoon JB. 1987. An outbreak of viral haemorrhagic pneumonia (tentative name) of rabbits in Korea. J Korean Vet Med Assoc 23:603–610.

23. Rahali N, Sghaier S, Kbaier H, Zanati A, Bahloul C. Genetic characterization and phylogenetic analysis of rabbit hemorrhagic disease virus isolated in Tunisia from 2015 to 2018. Arch Virol. 2019 Sep;164(9):2327-2332.
24. Rocchi M, Maley M, Dagleish M, Boag B. Rabbit haemorrhagic disease virus type 2 in hares in Scotland. Vet Rec. 2019;185(1):23.
25. Rouco C, Aguayo-Adán JA, Santoro S, Abrantes J, Delibes-Mateos M. Worldwide rapid spread of the novel rabbit hemorrhagic disease virus (GI.2/RHDV2/b). Transbound Emerg Dis. 2019;66(4):1762-1764.
26. Velarde R, Cavadini P, Neimanis A, Cabezón O, Chiari M, Gaffuri A, Lavín S, Grilli G, Gavier-Widén D, Lavazza A, Capucci L. 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. 2017 Dec;64(6):1750-1761.

Figure 1. (A) Detection of Calicivirus-like particles by electron microscopy in 10% liver homogenates from 2016 Quebec submission (NCFAD Lab # WIN-AH-2016-OTH-0018). (B) Detection of RHDV specific amplicons by conventional PCR. Lane 1 : 50 bp molecular weight marker. Lanes 2: Spleen 3: Liver, 4: Kidney, 5: Lung , 6: Liver, 7, 9: Extraction controls and 10: No template controls, 8: Positive control (RHDV1 isolate Mexico/2718, GenBank accession # KY235677).

Figure 2. Gross pathology findings in rabbits inoculated with RHDV2 strain from British Columbia (NCFAD Lab # WIN-AH-2018-OTH-0024). (A) Enhanced zonal pattern reflecting a periportal distribution of necrosis in the liver. (B) Liver is enlarged and pale with rounded edges and a fissure (arrow). (C) Lung: Multifocal hemorrhages (arrows). (D) Heart: Multifocal epicardial hemorrhages (arrows)

Figure 3. Microscopic lesions and detection of viral RNA by in situ hybridization in liver, kidney spleen, Lung and Heart of rabbits experimentally infected with RHDV2 strain from British Columbia (NCFAD Lab # WIN-AH-2018-OTH-0024). A. Liver: Widespread hepatic necrosis is found in a primarily periportal distribution (area of necrosis outlined by arrowheads) with relative sparing of the centrilobular areas (*). B. Liver: Extensive staining for viral RNA within the periportal areas (*) with only scattered single positive cells in the centrilobular area (arrowhead). C. Kidney: Microthrombi are in many of the glomeruli (arrows). D. Kidney: Presence of viral RNA in glomeruli (arrowheads) as well as interstitial blood vessels (arrow). E. Spleen: Red pulp areas contain abundant fibrin (arrows); there are scattered necrotic cells (arrowheads). There is lymphocytolysis and hemorrhage in white pulp areas (*). F. Spleen: There

414 is abundant positive staining for viral RNA throughout both red and white pulp. G. Lung:
415 Alveolar hemorrhage and edema. H. Lung: Abundant staining for viral RNA within alveolar
416 septae. I. Heart: Focal myocardial hemorrhage and a microthrombus (arrow). J. Heart: Detection
417 of viral RNA appears to be limited to the intercellular capillaries. A-E bar= 50 µm; F bar=100
418 µm.
419
420 **Figure 4.** 4a. Maximum-likelihood (ML) phylogenetic tree of the VP60 region of various
421 isolates of Rabbit hemorrhagic disease virus (RHDV). The sequences were directly downloaded
422 from or were extracted from the whole genome sequences downloaded from the GenBank
423 (https://www.ncbi.nlm.nih.gov/genbank/). Sequences were aligned in Geneious Prime
424 (Biomatters Inc.,San Diego, CA)., followed by phylogenetic tree construction using the IQ-
425 TREE Web server (http://iqtree.cibiv.univie.ac.at/), using the GTR+F+I+G4 nucleotide
426 substitution model and 1000 bootstrap replicates as indicated in the tree. The phylogenetic tree
427 was then visualized using iTOL (https://itol.embl.de/). Canadian isolates submitted to the
428 NCFAD are highlighted in blue. Genotypes are indicated on the right. 4b. Maximum-likelihood
429 (ML) phylogenetic tree of the whole genome sequences of various isolates of rabbit hemorrhagic
430 disease virus (RHDV). Whole genome sequences were downloaded from Genbank and were
431 aligned in Geneious Prime (v. 2020.1.1), followed by phylogenetic tree construction using the
432 IQ-TREE Web server, using the SYM+I+G4 nucleotide substitution model and 1000 bootstrap
433 replicates as indicated in the tree. The phylogenetic tree was then visualized using iTOL.
434 Canadian isolates submitted to the NCFAD are highlighted in blue. Genotypes are indicated on
435 the right. For clarity, only a limited number of RHDV1 whole genome sequences are depicted.