



NOTE

Pathology

An outbreak of rabbit hemorrhagic disease (RHD) caused by *Lagovirus europaeus* GI.2/rabbit hemorrhagic disease virus 2 (RHDV2) in Ehime, Japan

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ABSTRACT. A total of ten 1–2-year-old rabbits died within 2 weeks at a facility in Ehime prefecture in May 2019. Necropsy revealed liver discoloration and fragility, hemorrhage of some organs and blood coagulation failure. On histopathologic examination, necrotizing hepatitis was a common finding, together with fibrin thrombi in the small vessels and hemorrhage in some organs. Rabbit hemorrhagic disease (RHD) virus gene was detected in liver samples, and viral particles of approximately 32 nm in diameter were found in the cytoplasm of degenerated hepatocytes by electron microscopy. Phylogenetic analysis based on the partial VP60 gene sequence classified it as *Lagovirus europaeus* GI.2/RHDV2. This is the first confirmed outbreak of RHD caused by globally emerging GI.2/RHDV2 in Japan.

KEY WORDS: electron microscopy, Japan, *Lagovirus europaeus* GI.2/RHDV2, rabbit hemorrhagic disease (RHD)

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Rabbit hemorrhagic disease (RHD) is a highly contagious fatal disease in rabbits (*Oryctolagus cuniculus*) that was first reported in China in 1984 [1, 3]. RHD has rapidly spread worldwide, including Europe and other continents [3]. The first outbreak of RHD in Japan was recorded in 1994 in Hokkaido, with the most recent occurrence in 2002 [6, 12]. The causative agent is RHD virus (RHDV), a member of the *Caliciviridae* family and *Lagovirus* genus. A new variant of RHDV, RHDV2 or RHDVb, affecting not only *O. cuniculus* but also some *Lepus* spp., was identified in France in 2010 [8, 9]. Currently, RHDV2 has spread globally and affects pet and farmed or wild rabbits and hares [17]. RHDV and related viruses were re-classified as *Lagovirus europaeus* GI.1 (classical RHDV), GI.2 (RHDV2/b), GI.3, GI.4, GII.1 and GII.2, based on full-genome analysis [10]. Here, we describe the RHDVs classified as *Lagovirus europaeus* GI.2 as RHDV2. After a 17-year absence in Japan, an outbreak of RHD occurred in May 2019, in which RHDV2 was identified as the causative agent. This report describes the details of this outbreak.

Eight 1–2-year-old rabbits (*O. cuniculus*) in the same breeding room at an exhibition facility in Ehime prefecture all died within 1 week in May 2019. Most died suddenly, without presenting any clinical symptoms. Some showed a loss of appetite and respiratory distress and died during the acute course. The other two rabbits, which had been kept in a quarantine room, died after one of the eight rabbits were isolated in the quarantine room after the onset of clinical signs. Six of the ten dead rabbits were subjected to necropsy for histopathological and virological examinations. Necropsy revealed liver discoloration and fragility, hemorrhage of the lung, blood-filled fluid in the trachea, congestion and hemorrhage of the kidney, congestion of the spleen, and blood coagulation failure (Fig. 1).

Tissue samples for histopathological examination were fixed in 10% neutral buffered formalin, routinely paraffin-embedded, and sectioned at 3–4 μm for hematoxylin and eosin (HE) staining. The formalin-fixed livers were trimmed into small pieces, rinsed with phosphate-buffered saline, post-fixed with 1% osmium tetroxide, and embedded in epoxy resin for electron microscopy examination. Ultrathin sections were double-stained with uranyl acetate and lead citrate and examined using a transmission electron microscope (JEM-1400Flash, JEM-1010; JEOL Ltd., Tokyo, Japan). Total RNA was extracted from liver homogenates by TRIzol LS reagent (Thermo Fisher Scientific, Carlsbad, CA, USA) and tested for RHDV using a Takara PrimeScript OneStep RT-PCR Kit ver.2 (Takara, Tokyo, Japan) with two primer sets targeting non-structural protein (NSP) (p29 and p30) [7] and capsid

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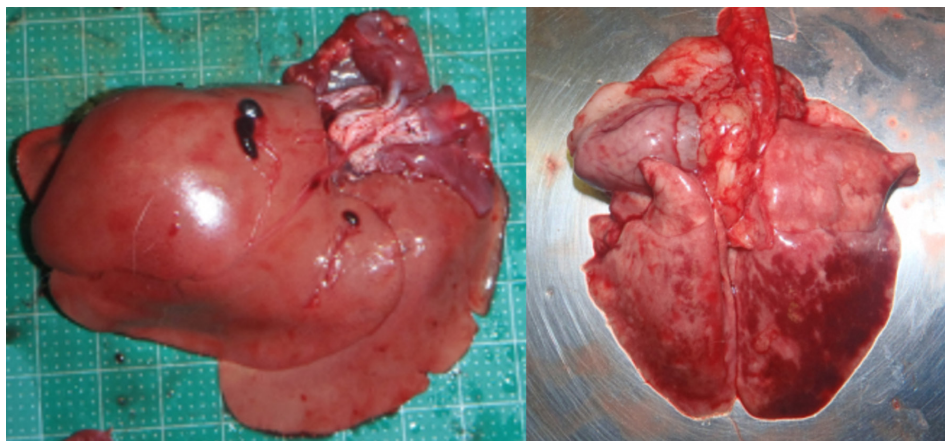


Fig. 1. Macroscopic findings of the necropsied rabbits. Discoloration of the liver (left) and hemorrhage of the lung (right).

protein VP60 gene (14U1 and RVP60-L1) [9, 19]. Samples from past RHD outbreaks in Japan (Tokyo-1/2000, Tokyo-2/2000, Hokkaido-3/2002 and Hokkaido-4/2002) were also included as positive controls. The RT-PCR products with the expected amplicon sizes were purified and directly sequenced from both directions. The sequences were assembled, edited, and analyzed using MEGA 7 software and compared to those of reference strains available in GenBank. Alignments of multiple sequences were performed using CLUSTAL W and phylogenetic trees were constructed using the Maximum Likelihood method based on the Tamura-Nei model with 1,000 bootstrap replications in MEGA 7 software. Nucleotide sequences determined in this study were deposited in DNA Data Bank of Japan (DDBJ accession numbers LC611474-LC611479).

Histopathological examination revealed peripheral to midzonal necrosis in the livers, which was a common finding in necropsied rabbits, sometimes with mild hemorrhage and heterophil infiltration (Fig. 2). The cytoplasm of necrotizing hepatocytes became increasingly eosinophilic with occasional karyopycnosis or karyorrhexis. Mild infiltration of lymphocytes, plasma cells, and macrophages was also observed in the portal area. Several rabbits also showed lymphocyte depletion in the spleen, mild to moderate hemorrhage in the alveoli of the lung, and fibrin thrombi in the small blood vessels or capillaries in the kidneys and lungs. Viral particles approximately 32 nm in diameter were found in the cytoplasm of degenerated hepatocytes by electron microscopy (Fig. 3). Some viral particles were present along the membrane-like structure. RHDV NSP and VP60 genes were detected in the livers of all six necropsied animals. The RT-PCR products from two of the six rabbits (Ehime-1 and Ehime-2) were directly sequenced for further genetic analysis. The 442- and 770-nucleotide partial sequences of NSP and VP60 gene from Ehime-1 and Ehime-2 were 100% identical to each other, respectively. BLAST analysis showed that the partial NSP and VP60 sequences detected in Ehime-1 had the highest similarity to those of RHDV2 reported in Australia in 2016, with 99.6% (AUS/WA/WNP-1/2016) and 99.2% (AUS/WA/PTH-3/2016) identity, respectively. Phylogenetic tree constructed based on 746 nucleotides from VP60 gene demonstrated that the Ehime-1 and Ehime-2 sequences were closely related to those of RHDV2 strains but not to those of the past Japanese RHD cases (Fig. 4).

This report describes the first confirmed cases of RHD caused by RHDV2 in Japan. After this outbreak, a total of 45 affected rabbits in 8 outbreaks were reported in 5 prefectures by August 2020 [13]. Since its appearance in France in 2010, RHDV2 has quickly spread worldwide and appears to have replaced the classical RHDV [17]. The mortality rate of RHD caused by RHDV2 is lower than that of classical RHDV but appears highly variable, ranging from 9% to 46% depending on strains [9]. Recent reports showed that two Italian RHDV2 strains isolated in 2014 and 2015 induced at least 80% mortality, which approaches the usual mortality rate of classical RHDV [2]. Experimental infection with the Australian isolate of RHDV2 resulted in high mortality rate [14]. RHDV2 strains with higher pathogenicity may have emerged and become prevalent in the field instead of the early strains reported in France [2, 14]. The RHDV2 strain in the present case also seems to induce a high mortality rate. Phylogenetic analysis demonstrated that RHDV sequences detected in the present case were genetically related to those of the Australian RHDV2 strains, although the route of entry was unknown epidemiologically.

The pathological findings were similar to those of typical RHD caused by classical RHDV and RHDV2 [1, 3, 5, 14, 16, 20], including necrotizing hepatitis, disseminated intravascular coagulation and hemorrhage in the organs. Decrease in number of lymphocytes was also observed in lymphatic organs. The viral particle of RHDV is 32–40 nm in diameter [1, 3, 15, 19]. Although a few reports have described the results of electron microscope examination in field cases of RHD, our findings were consistent with the observation by Park *et al.* [15]. Viral particles were sometimes observed along the membrane-like structure, although the detailed structure of the organelles was unclear in degenerated hepatocytes.

The impact of RHDV2 may be more serious than that of classical RHDV because RHDV2 can affect young rabbits under 1 month of age, unlike classical RHDV [11, 14], and affects not only rabbits (*O. cuniculus*) but also hares (*Lepus* spp.) [4, 18]. To date, there are no commercially available RHD vaccines in Japan. Considering the stability of RHDV under environmental conditions [16, 20], biosecurity protocols such as daily disinfection and health monitoring should be implemented in rabbit-rearing facilities. In addition, the spread of RHDV2 to wild hares in Japan is of great concern and requires a close watch on the situation.

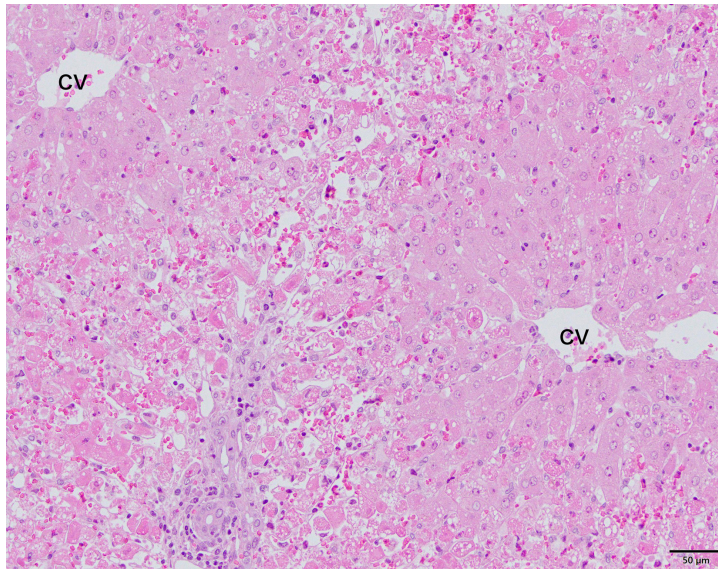


Fig. 2. Peripheral to midzonal necrosis in the liver with infiltration of a small number of inflammatory cells. CV: central vein. Hematoxylin and eosin. Bar=50 μm.

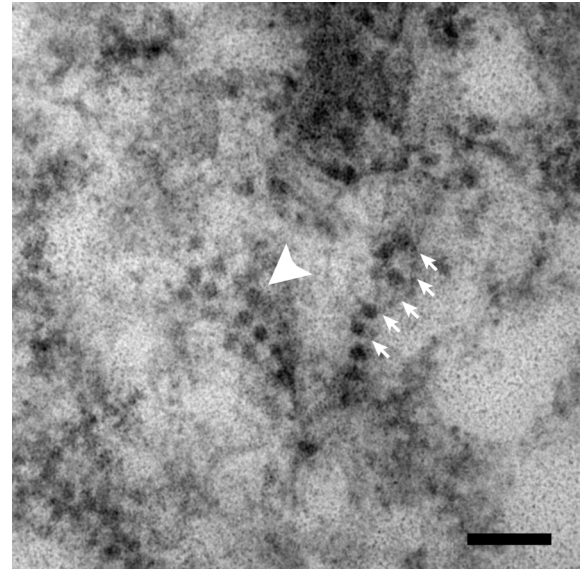


Fig. 3. Viral particles in the degenerated hepatocyte (arrow-head). The viral particle measured approximately 32 nm in diameter. Membrane-like structure is shown by arrows. Transmission electron microscopy, double-stained with uranyl acetate and lead citrate. Bar=100 nm.

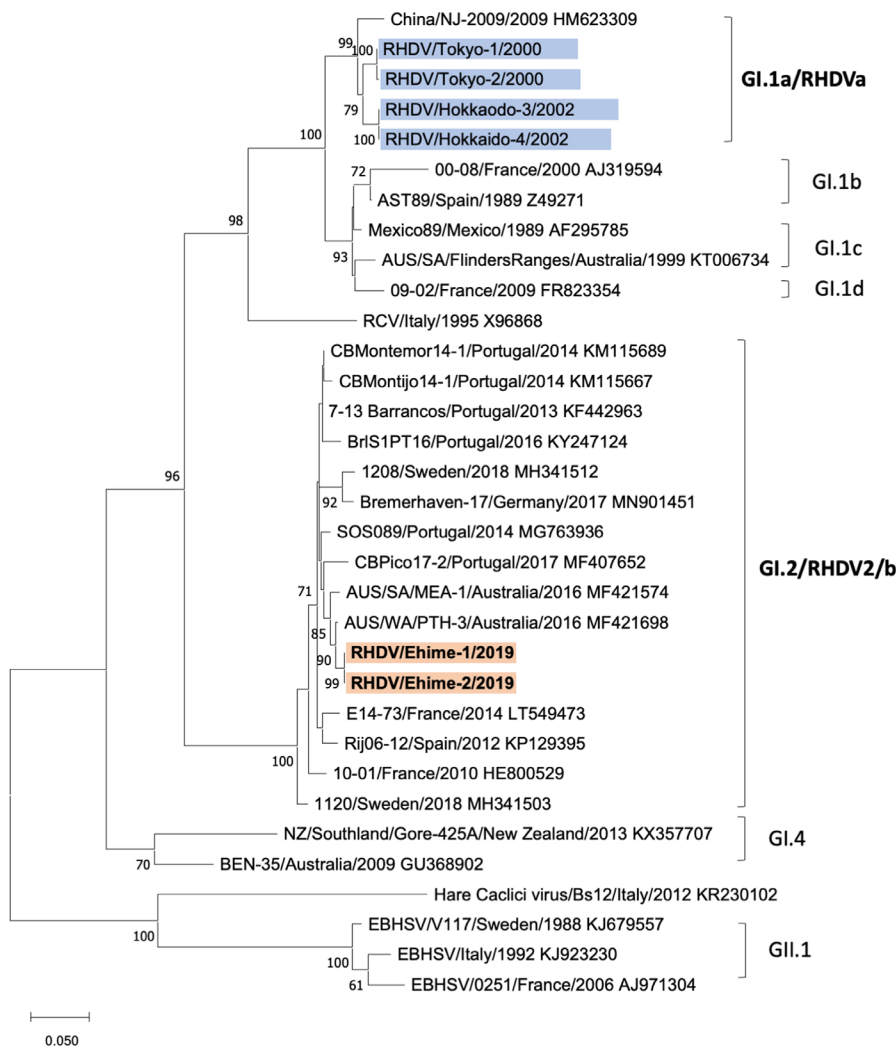


Fig. 4. Phylogenetic tree based on the partial VP60 gene sequence of rabbit hemorrhagic disease virus (RHDV) using the Maximum Likelihood method based on the Tamura-Nei model with 1,000 bootstrap replications in MEGA 7 software. Bootstrap values above 60% are shown at the branch node. The sequences determined in samples from the current case (RHDV/Ehime-1/2019 and RHDV/Ehime-2/2019) and past cases in Japan (Tokyo-1/2000, Tokyo-2/2000, Hokkaido-3/2002, and Hokkaido-4/2002) are highlighted in red and blue, respectively.

CONFLICT OF INTEREST. The authors have no conflicts of interest to declare.

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