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NOTE

Pathology

Rabbit hemorrhagic disease virus type 2 epidemic in a rabbit colony in Japan

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ABSTRACT. Twenty-three of 42 European rabbits (*Oryctolagus cuniculus*), belonging to the same rabbit colony, died in March 2020 (55% mortality) in Chiba prefecture, Japan. The disease course was extremely acute without indicators of death or hemorrhage. Necropsy revealed liver swelling, discoloration, cloudiness and fragility, and pulmonary edema. Histologically, severe hepatocellular necrosis (mainly peripheral) and intra-glomerular capillary hyalin thrombi were observed. On molecular-biological examination, reverse transcription polymerase chain reaction analysis of RNA from tissues detected a rabbit hemorrhagic disease virus, confirmed as a RHDV-2 VP60 fragment, which shared 99.42% nucleotide identity with the homologous fragment of RHDV-2 German isolate by nucleotide sequence analysis. This report shows the outbreak of rabbit hemorrhagic disease caused by RHDV-2, an emerging infectious disease, in Japan.

KEY WORDS: European rabbit, Japan, rabbit hemorrhagic disease, rabbit hemorrhagic disease virus type 2

Rabbit hemorrhagic disease (RHD) is caused by rabbit hemorrhagic disease virus (RHDV), belonging to the genus *Lagovirus*, family *Caliciviriade* [12]. RHDV causes highly contagious, acute, and fatal hepatitis in wild and domestic European rabbits (*Oryctolagus cuniculus*) [1]. In Japan, RHD is designated as a monitored infectious disease (Act on Domestic Animal Infectious Diseases Control). When identified, there is a legal requirement to notify the prefectural governor through the Livestock Hygiene Service Center [7]. Lagovirus contains two genogroups that can be subdivided into genotypes, further subdivided into variants [8]. RHDV is classified into genotype GI.1 ("classical RHDV") and GI.2 (RHDV-2), which was first isolated in France in 2010. The nucleotide diversity of the capsid protein (VP60) of RHDV-2 differs from "classical RHDV" by more than 15%. RHDV-2 has spread throughout Europe and replaced circulating "classical RHDV" strains in most European countries [1, 6]. Also, epidemics of RHDV-2 have been reported frequently in the United States [16]. Here, we describe the outbreak of RHD caused by RHDV-2 in Japan.

In March 2020, 23 of 42 rabbits died in a 1-month period, within a zoological garden in Chiba prefecture (mortality rate 55%). The clinical course was extremely acute, with no apparent signs of death or hemorrhage. Surviving rabbits varied in age but did not include animals younger than 2 months. These rabbits had come into contact with many visitors. Rabbits were mainly fed commercial pellets and vegetables such as carrots and, occasionally, wild grasses. Since they shared a ranch with other herbivores, they were sometimes fed purchased herbage. No new rabbits were introduced since the acceptance of 3 rabbits on February 25, 2018, but construction work was carried out to expand the ranch before the RHD epidemic.

The facility requested our laboratory to determine the cause of death of the rabbits. Three rabbits that died during the specified period were necropsied. All visceral organs were fixed using 10% buffered formalin solution for histopathological examination, and the liver, lungs, spleen, and kidneys were collected without any fixation for viral examination. RHD was suspected and further tests confirmed the presence of the virus. Rabbit 1 was male and sub-adult and weighed 1,850 g. Rabbit 2 was female and sub-adult and weighed 975 g. Rabbit 3 was a lactating female adult that weighed 1,766 g. Macroscopic examination of the body condition of each rabbit revealed a physical appearance indicative of good health with well-developed body fat and a full stomach. Cyanosis of the oral mucosa was observed in all rabbits. Further, rabbits 1 and 3 had severely enlarged livers that were discolored, cloudy, and fragile (Figs. 1 and 2). The liver of each rabbit showed a clear lobular structure. The liver of rabbit 2 showed moderate swelling and congestion. Each of the three rabbits had varying degrees of pulmonary edema. The three-layer structure within the kidney of each rabbit was unclear. No marked splenomegaly was observed, and follicles and splenic trabecula were inconspicuous.

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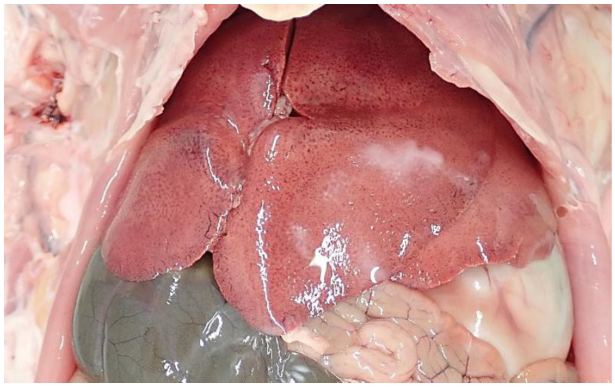

Fig. 1. Macroscopic features of the visceral organs of rabbit 3. The liver was severely enlarged, discolored, and cloudy. No hemorrhage was observed in the abdominal organs.

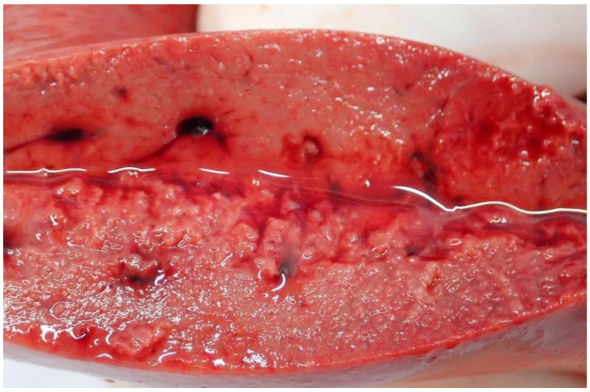

Fig. 2. Macroscopic findings in rabbit 1. The liver was cloudy and very fragile.

There was no swelling of the lymph nodes, and no obvious hemorrhage was observed.

Histopathologically, there were no clear differences in the quality and distribution of lesions observed in the three individual rabbits. Significant degeneration and necrosis were seen in the liver. Hepatic necrosis was severe in the periportal area, with extensive and sometimes band-like necrosis. In severe cases (rabbits 1 and 3), necrosis was widespread throughout the hepatic lobule and few non-necrotic hepatocytes were observed (Fig. 3). Affected hepatocytes had hypereosinophilic cytoplasm and typically karyorrhectic or pyknotic nuclei. In advanced stages of the hepatic lesion, cells or cell fragments were increasingly rounded, diminished in size, often contained pyknotic nuclear fragments, and were detached from hepatic cords (consistent with characteristics of apoptotic cells and apoptotic bodies). Fibrin and hemorrhage were seen in the area where hepatocytes collapsed and disappeared. Various degrees of vacuolar degeneration were observed in the remaining hepatocytes. In parts with severe degeneration in rabbit 1, aggregates of various forms of eosinophilic material, sometimes with a crystalline structure, were observed in balloon-like swollen hepatocytes (Fig. 4). This intra-cytoplasmic inclusion body was stained red by Azan staining, blue by phosphotungstic acid-hematoxylin (PTAH) staining, light pink by periodic acid-Schiff reaction, and indistinctly by pyronin-methyl green staining. Overall, inflammatory cell infiltration was poor. In the affected kidneys, a hyalin-like substance was found in the renal glomerular capillaries of all rabbits (Fig. 5). The material suspected hyaline thrombus appeared light blue as a result of PTAH staining. Degeneration and necrosis of the tubular epithelium were also observed. Pulmonary edema was prominent. Both ventricular dilatations and myocardial degeneration (eosinophilic, hydropic, and vacuolar degeneration) were observed in all rabbits. In addition, rabbit 2 had multiple focal, myocardial micronecrosis. In the lymph nodes, lymphocyte necrosis was only found in rabbit 2. Microscopic hemorrhage was observed in the liver, kidneys, spleen, heart, and lungs of all the three rabbits. No nuclear inclusions were observed in any of the cells, including hepatocytes.

Total RNA was extracted from each sample (liver, lung, spleen, and kidney) from each of the three rabbits using a QIAamp Viral RNA Mini Kit (QIAGEN, Inc., Valencia, CA, USA), in accordance with the manufacturer's instructions. A 398-bp region of the *VP60* (capsid) gene was initially targeted using RHDV-specific primers [2]. Reverse transcription polymerase chain reaction (RT-PCR) was performed using a One-Step RT-PCR Kit (QIAGEN) with forward and reverse primers as follows: forward, 5′-GTT ACG ACT GTG CAG GCC TAT GAG TT-3′; and reverse, 5′-TTG TTG AGC AGT CCA ATT GTC ACT G-3′ [3].

As a result, RHDV capsid-specific PCR amplification product was obtained from all samples. Nucleotide sequence analysis using 344 bp (nucleotide position: 6429–6772) of the PCR product from all liver samples revealed the presence of RHDV-type 2 viruses, which shared highest homology of 99.42% with the German strain (LR899153) (Fig. 6). Virus isolation was attempted using Vero, BHK, fcwf-4, CRFK, MDCK, and rabbit kidney-derived LCC-RK1 (JCRB9034) cell cultures, but the attempts were unsuccessful.

We diagnosed the rabbits with RHD, caused by RHDV-2, based on the identified pathological features and molecular assay for viral detection and analyses. This is the first report on the occurrence of RHD caused by RHDV-2 in Japan to the best of our knowledge without the published report. A presumptive diagnosis of RHD is often made based on clinical symptoms, the infection pattern within a population, and post-mortem lesion assessment [5]. RHD is suspected if sudden death occurs over a short period and liver injury is observed, even if hemorrhage, a characteristic of RHD, is not noticeable.

Available data suggest that RHDV-2 has recently emerged, and its origin is distinct from that of classic RHDV [6]. Clinical and epidemical characteristics of classic RHDV and RHDV-2 also differ [5]. Classic RHDV has an incubation period of 1–3 days and an acute course that results in death in 12–36 hr after fever. The mortality rate of classic RHDV is almost 100%, and rabbits younger than 6–8 weeks are asymptomatic. In comparison, the clinical course of RHDV-2 type disease is longer (3–5 days), and the mortality rate varies from 5% to 70% [13]. Furthermore, rabbits of all ages can be affected. Here, the reported mortality rate of 55% and the presence of microscopically observable hemorrhage were attributed to the identified virus type. Histopathological findings of this epidemic were consistent with those of RHD common to classic RHDV [10] and RHDV-2 [13]. In RHD, inclusion

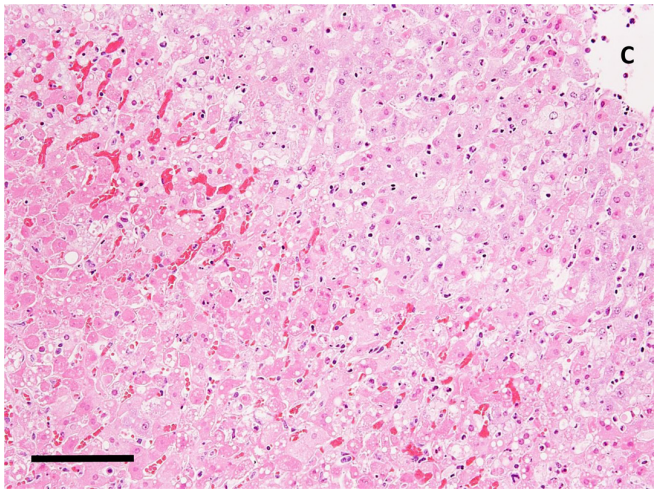

Fig. 3. Microscopic features of the liver in rabbit 2. Hepatic necrosis was extensive and occurred primarily in periportal areas. Scale bar: 100 µm. C=central vein.

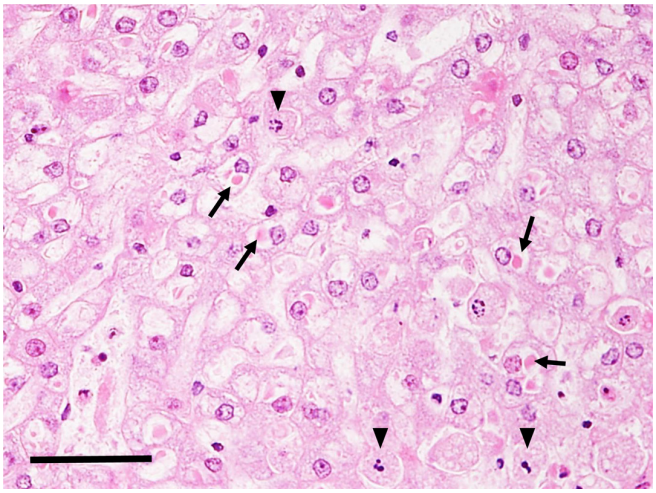

Fig. 4. Microscopic features of the liver in rabbit 1. Eosinophilic intracytoplasmic inclusions (arrows) and apoptosis corpuscles (arrowheads) were observed in hepatocytes. Scale bar: 50 µm.

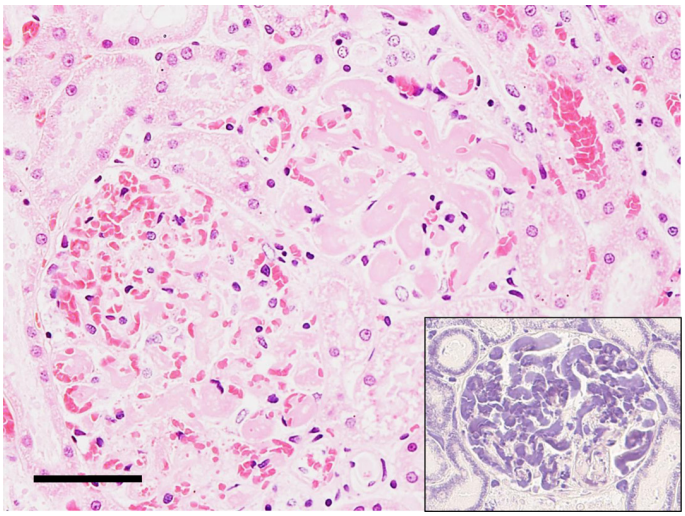

Fig. 5. Microscopic features of the kidney of rabbit 1. Hyalin-like substance was found in the renal glomerular capillaries. Scale bar: 50 µm. Insert: phosphotungstic acid-hematoxylin staining.

bodies are reportedly found in the nucleus of hepatocytes and spleen cells [4]. However, they were not observed in our case. Intra-cytoplasmic inclusion bodies were observed in hepatic phagocytes–Kupffer cells in rabbits experimentally inoculated with RHD [14]. Intra-cytoplasmic eosinophilic inclusion bodies of hepatocytes have also been reported in RHDV-2 [11], and the precise contents of such bodies are unknown. Since inclusion bodies are found in highly degenerated hepatocytes and appear crystalline, it is likely that they consist of degenerated substances.

Rabbit calicivirus is highly infectious, is highly resistant to breakdown in the environment, and is transmitted by direct or indirect contact with infected animals, carcasses, body fluids (urine, feces, respiratory secretions), and fur [5]. At the facility affected by the outbreak, since no new animals were introduced, indirect contact with the source of infection via humans or materials was suspected. A limitation of the study is that the source of infection could not be confirmed. Although contaminated herbage was suspected to be the infection source, no further investigation was carried out. Since the outbreak, RHD has occurred in six episodes and five prefectures throughout the Kanto and Tohoku regions, and a total of 20 rabbits have been affected from May to August 2020 [9]. Thus, special monitoring is required, and the identification of each infection source should be considered a priority.

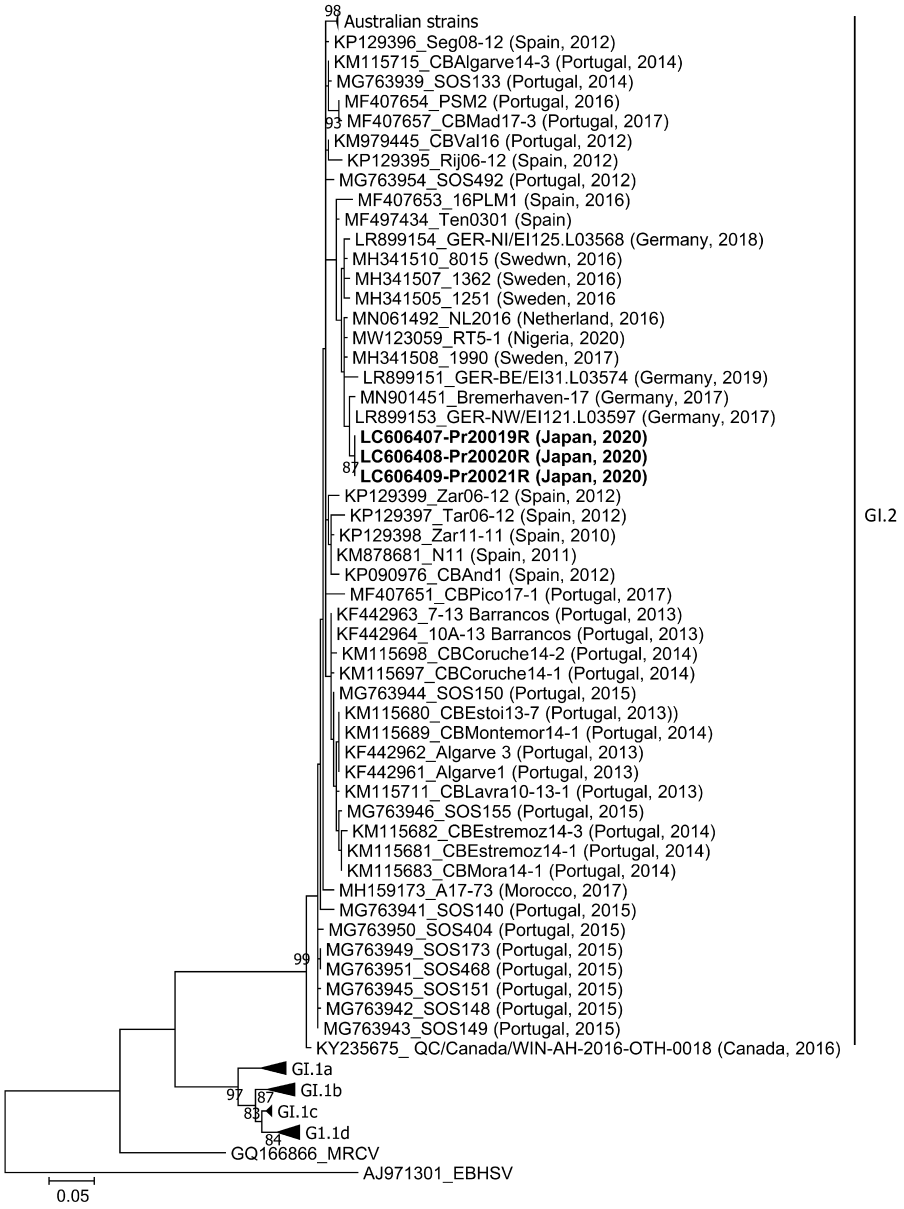

Fig. 6. Phylogenetic analysis of VP60 gene in GI lagoviruses. A phylogenetic tree was constructed using the Maximum Likelihood method based on the Tamura-Nei model [15] on a partial VP60 gene (nucleotide position: 6429–6772) of a representative subset of published lagoviruses (n=93) and detected gene in the present study. Accession numbers of all sequence genomes from GenBank, their strain names, origin countries, and collected date (in parenthesis) are indicated in the taxon names. The newly sequenced strains are shown in boldface. The genotype GI.1a, GI.1b, GI.1c, GI.1d, and Australian GI.2 clades are collapsed due to their large number. Michigan rabbit calicivirus (MRCV) and European brown hare syndrome virus (EBHSV) are used as the outgroup to root the tree. The percentage of trees in which the associated taxa clustered together is shown next to the branches. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site.

CONFLICT OF INTEREST. The authors declare no conflict of interest.

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