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Detection of a new emerging strain of rabbit haemorrhagic disease virus 2 (GI.2) in China

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Abstract

Introduction: In May 2020, an outbreak of rabbit haemorrhagic disease 2 (RHD2) caused by the rabbit haemorrhagic disease virus 2 (RHDV2, GI.2) occurred in Sichuan, China. The acute onset and short disease course resulted in rabbit mortality as high as 42.86%. Currently, basic research on the aetiology and genetic characteristics of GI.2 is lacking in China. **Material and Methods:** Pathological changes in various tissues from infected rabbits were investigated and the viral genome was characterised. This study used RT-PCR, histopathology and scanning electron microscopy to identify the pathogen in samples from infected rabbits that had died. Phylogenetic trees were constructed based on whole genome sequence analysis, and recombination events were analysed. **Results:** RT-PCR identified the presence of GI.2. Histopathology revealed liver cell necrosis and haemorrhaging into lung alveoli. Electron microscopy demonstrated spherical GI.2 particles that were 40 nm in size. The gene sequence length of the isolate was 7,445 bp (GenBank accession number MW178244). A phylogenetic analysis based on the genome of the isolated strain and 60 reference strains showed that the isolate was grouped together with GI.2 strain MT586027.1 in a relatively independent sub-branch. The results of the recombination analysis showed that the strain was recombined from the MT586027.1 (major parent) and MN90145.1 (minor parent) strains, and recombination breakpoints were at locations in the 2858–5137 nt range. **Conclusion:** The results of this study extend our understanding of the molecular epidemiology of GI.2.

Keywords: China, rabbit haemorrhagic disease virus 2, whole genome sequence, genetic variation.

Introduction

Rabbit haemorrhagic disease (RHD), also known as rabbit plague and rabbit haemorrhagic pneumonia, is caused by the rabbit haemorrhagic disease virus (RHDV), which belongs to the Caliciviridae family and *Lagovirus* genus (23). It is an acute, septic, highly lethal and infectious disorder. The disease caused by RHDV first broke out in Wuxi, Jiangsu, and other locations in China in 1984 (14). Young rabbits older than two months of age and adult rabbits are susceptible to classic RHD and among them the mortality rate is 80–90%. In 2010, a new RHDV variant was identified affecting wild and farmed rabbits in France (12). The virus, designated RHDV2, was considered a distinct serotype as it had a sufficiently different antigenic profile from that of the original RHDV. Consequent upon the 2017 attempt to standardise the *Lagovirus* nomenclature, RHDV belongs to the GI genogroup, which is further divided into the GI.1 (former G1–G6), GI.2 (RHDV2/b), GI.3 (non-pathogenic rabbit calicivirus E-1) and GI.4 (non-pathogenic rabbit calicivirus A-1/E-2) genogroups (13). While GI.1 RHDV exclusively kills adults, GI.2 also kills sub-adult rabbits and kits from 11 days old (7). Infected rabbits that die exhibit haemorrhaging and enlargement of the liver, spleen, kidney and other organs, as well as congestion in the nasal cavity and trachea (1, 18). Infection with GI.2 results in hepatic necrosis and diffuse intravascular coagulation, leading to substantial haemorrhaging (19). Molecular epidemiological studies have demonstrated that GI.2 has replaced GI.1 as the current epidemic strain in France, Spain, Portugal, Australia and other countries (6, 15). Under natural conditions, GI.2 infects rabbits and hares, but rabbits are more susceptible, especially young and juvenile individuals (3, 8). Water and food contaminated

with GI.2 facilitate the virus' infection of the host through the respiratory and digestive tracts by epithelial cell histo-blood group antigen attachment factors (20).

GI.2 has no envelope structure. The virus is spherical with a diameter of 32–44 nm, displays icosahedral symmetry and has single positive-stranded RNA and a genome of 7,442 bp. The virus is slightly different from GI.1 (9, 16, 21), and the nucleotide sequence of the two is only 82.4% homologous (12). The GI.2 genogroup genome has two open reading frames, ORF1 and ORF2. The first encodes a polyprotein, which is subdivided into multiple non-structural proteins, including p16; p23; p29; 2-chymotrypsin-like (2C-like) protein; 3C-like protein; virus protein, genome-linked (VPg); RNA-dependent RNA polymerase (RdRp); and capsid structural protein VP60 (7, 9). The second, and specifically its 3′-end guide RNA and single guide RNA, encodes the VP10 minor structural protein. Some studies have indicated that VP10 can increase the level of virus replication and promote cell apoptosis (14).

This research enriches the GI.2 gene sequence data which is documented and provides references for GI.2 diagnosis and for potential vaccine research and development using this genogroup. It also establishes a background for further research into the mechanisms of RHD to facilitate the development of new treatments.

Material and Methods

Sample collection and viral nucleic acid extraction. In April 2020, 100 dead rabbits 20 to 60 days old and suspected of being infected with GI.2 were collected from a rabbit farm in Jintang City, Sichuan Province, China. Under strict biosafety conditions, the dead rabbits were dissected and lesions were observed. Visceral tissues, including heart, liver, lung, kidney, spleen, lymph nodes, and any other tissues with apparent lesions, were collected and fixed in 4% paraformaldehyde for histopathology. All samples were tested for rabbit *Pasteurella*, European brown hare syndrome virus (25) and RHDV at the Sichuan Agricultural University (Chengdu, Sichuan, China).

Samples of the liver, lung, heart and spleen were homogenised, diluted tenfold with phosphate-buffered saline (PBS; 0.01 M, pH 7.4), and stored at −70°C until they were analysed. The samples were frozen and thawed three times to release the virus, then centrifuged at 8,000 rpm for 10 min at 4°C and the virus was collected from the supernatant. The viral genomic RNA was extracted using a total viral RNA extraction kit (TaKaRa, Beijing, China) and reverse transcribed into first-strand cDNA using M-MLV reverse transcriptase (TaKaRa). The cDNA was stored at −20°C for later use.

Genome amplification and sequencing. The cDNA obtained by reverse transcription was used as a template, and the primers for the GI.2 *VP60* gene were those established previously for an RT-PCR carried out in our laboratory (25) (forward 5′-GGGTGTCATATC CACCCCAAA-3′; and reverse 5′-CCCAGGTTGAAC ACGAG-3′). The target fragment was 441 bp. After the reaction, the PCR products were detected by electrophoresis (Bio-Rad, California, USA) using a 1% agarose gel (Solarbo, Beijing, China). The amplicons were gel purified and cloned, and recombinant plasmids of each clone were sent for sequencing to Sangon Biotech Co. (Shanghai, China). The sequencing results were compared and analysed with the use of the NCBI blastn tool.

Transmission electron microscopy (TEM). Liver tissue samples from the infected rabbits were homogenised in PBS, frozen and thawed three times to release the viral particles, then centrifuged at 8,000 rpm for 5 min. The resulting supernatant was used to create a virus solution, to which NaCl was added to produce a final concentration of 0.5 mol/L in order to precipitate the viral particles. An equal amount of 6% polyethylene glycol 6000 was added, and the solution was kept at 4°C overnight. The solution was centrifuged at 12,000 rpm for 1 h, the virus pellet was collected, and the supernatant was discarded. After adding an appropriate volume of PBS, the solution was centrifuged for 1 h at 12,000 rpm. The resulting pellet was washed twice and kept at 4°C. Subsequently, the sample was sent to Wuhan Servicebio Technology Co., Ltd. for further analysis by TEM.

GI.2 determination and sequencing of the full-length genome. The DNA library was sent to Shanghai Personalbio Biotechnology Co. (Shanghai, China) for high-throughput sequencing using Illumina NovaSeq (5) and the Bremerhaven-17 isolate detected in 2020 (GenBank accession number MN901451.1) as the reference genome. First, A5-MiSeq and SPAdes (2) were used to disassemble the sequencing data without the adapter sequence to construct a contig. The sequence was extracted according to the sequencing depth of the assembled sequence, and the sequence with high sequencing depth, and the viral genome sequence of each splicing result were selected. For the integration of splicing results, MUMmer software was used (11) to perform collinearity analysis to determine the positional relationship between contigs, and for filling gaps between contigs Pilon software was used (24), which corrects the results to obtain the final viral genome sequence.

Genome sequence analysis. Phylogenetic and molecular evolutionary analyses were conducted using MEGA version X (10). A bootstrap confidence value was calculated using 1,000 replicates.

Since the RHDV genome is prone to recombination, the dataset was screened for recombination with RDP software version 5 (17), with the following parameters: sequences were set to linear, Bonferroni correction, highest acceptable P-value of 0.05, and 100 permutations. Only recombination events detected by three or more methods were considered.

Results

Visceral lesions and nucleic acid test results obtained from rabbits which likely died of RHD. After examining the anatomy of the dead rabbits, bleeding spots or spots were observed in the lungs (Fig. 1A); tracheal cutting showed obvious bleeding and some to contain foam (Fig. 1B); the liver was fragile, enlarged, and haemorrhaged (Fig. 1C); most of the faeces were dry (Fig. 1E); urine was present in the bladder (Fig. 1F); and some rabbits had bled from the mouth and nose (Fig. 1D).

Histopathology. All animals displayed acute necrosis or apoptosis of individual hepatocytes that was characterised as severe and panlobular necrotising hepatitis with massive congestion and acute haemorrhage (Fig. 2 A and B). Splenic lymphocytes were necrotic, and there was haemorrhaging and congestion in the red pulp (Fig. 2C). Some lesions occurred in the alveoli. Some alveolar spaces were narrowed and exhibited mild haemorrhaging, while other alveolar spaces were completely filled (Fig. 2D). There were no apparent abnormalities observed in the heart or kidneys.

Transmission electron microscopy results. Examination of the GI.2 sample using TEM revealed a clear background with no viral envelope present. The electron microscopy results showed that the spherical viral particles have an icosahedral nucleocapsid diameter of 40 nm (Fig. 3).

Phylogenetic analysis of the complete GI.2 genome. Primers were used to amplify the *VP60* gene sequence. Agarose gel electrophoresis revealed a specific amplified band that was approximately 441 bp (Fig. 4). After the product was sequenced, it was analysed by BLAST, which indicated that the target fragment gene sequence was in GenBank. The sequence match for the GI.2 *VP60* gene (GenBank accession number MT586027.1) was 98.63%.

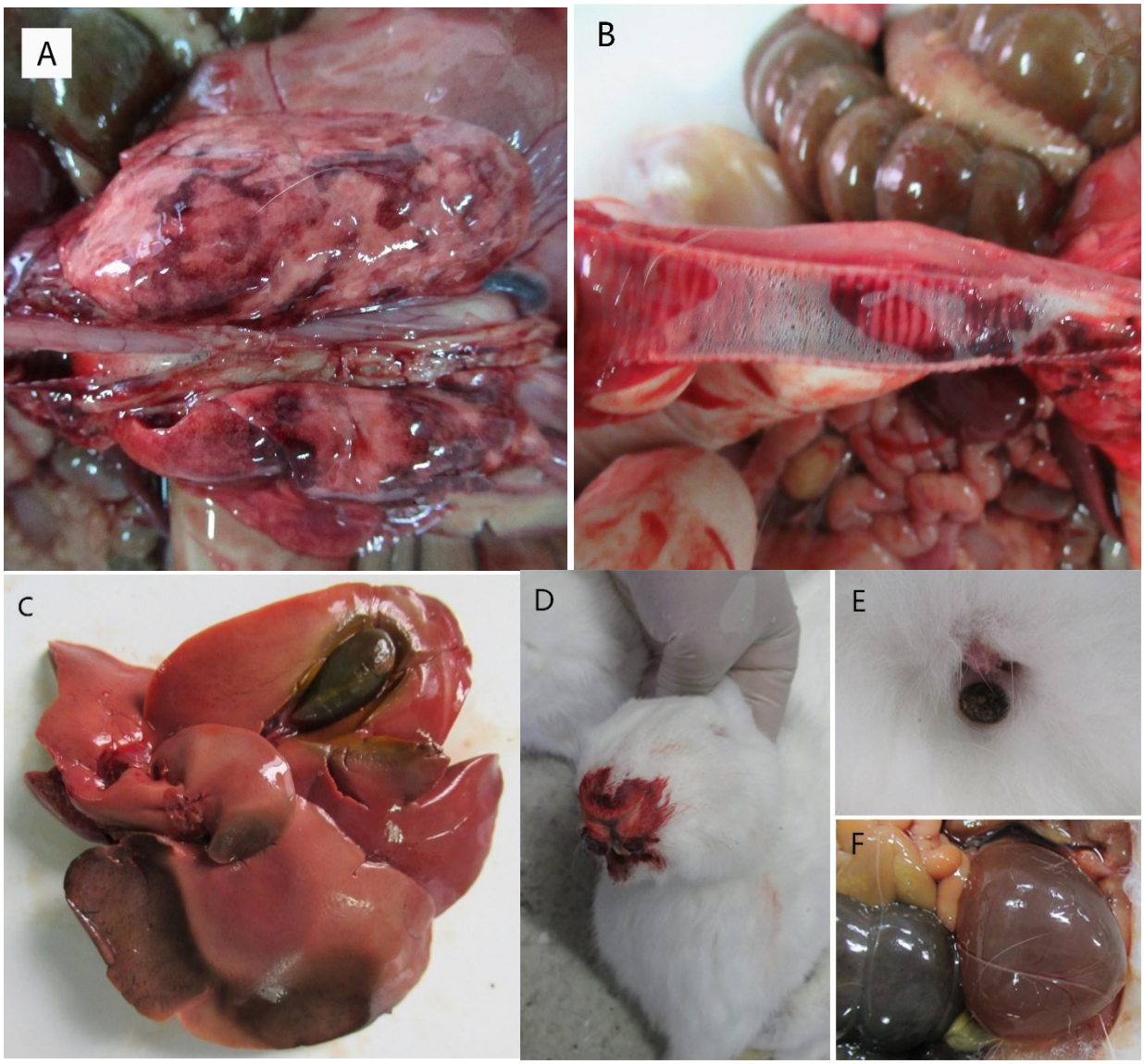

Fig. 1. Clinical pathological changes after GI.2 infection in rabbits. A – lungs and epicardium; B – tracheas; C – livers; D – facial appearance of an infected rabbit; E – faeces; F – bladders

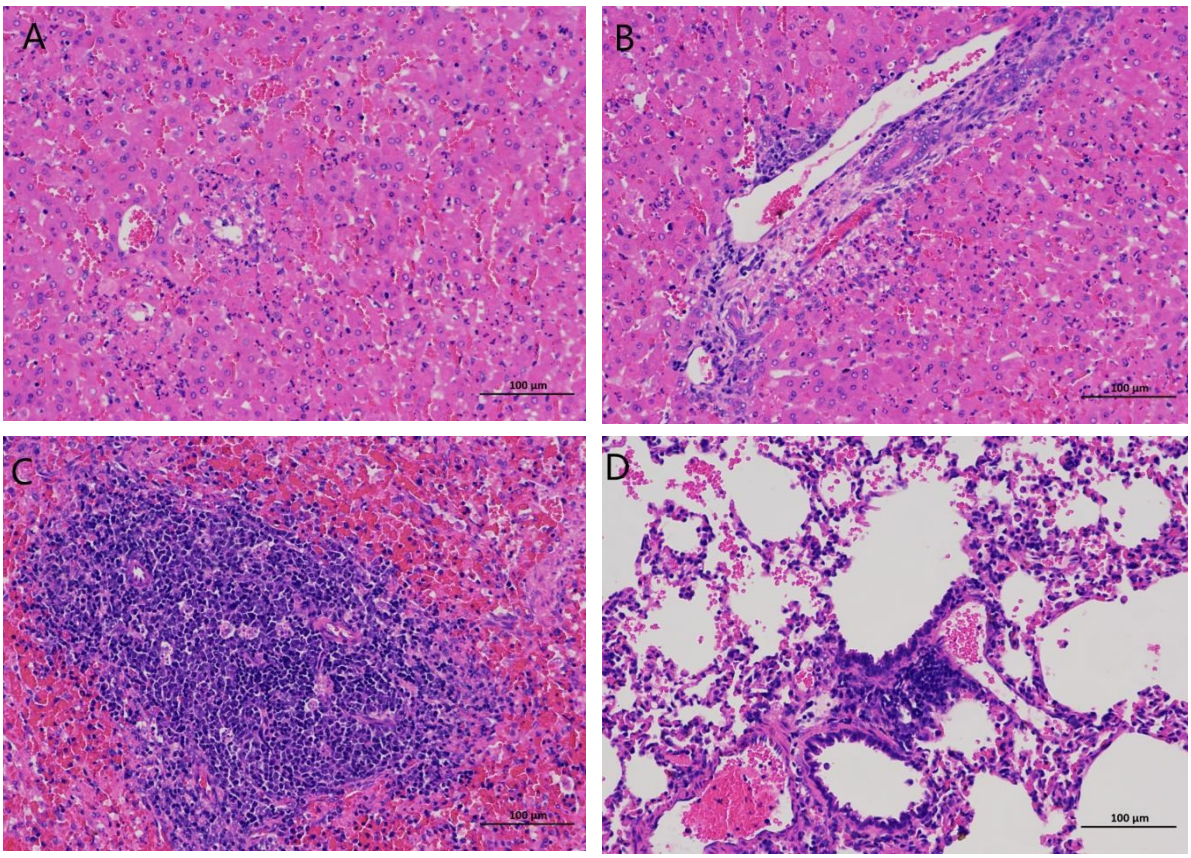

Fig. 2. Histopathological lesions in spleen, liver, and lung tissue. A and B – liver; C – spleen; D – lung. Scale bar 100µm

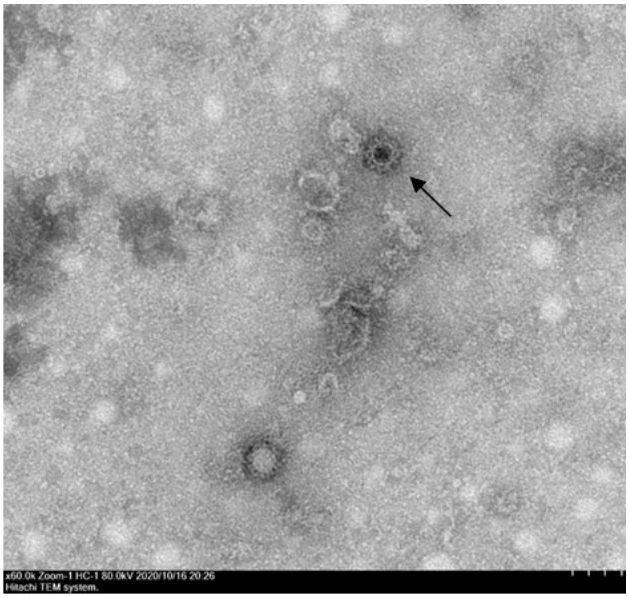

Fig. 3. Particles of the rabbit haemorrhagic disease virus (GI.2)

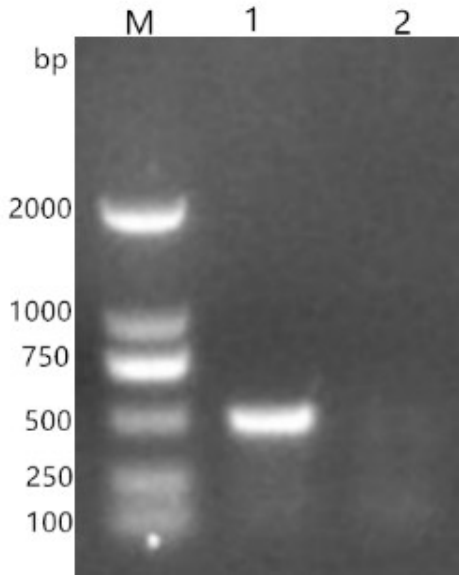

Fig. 4. PCR amplification results of the whole gene from the strain isolated in the present study. M – DNA marker; 1 – sample; 2 – negative control

The genome of GI.2 was amplified directly from tissue homogenates, and sequencing results showed it to have a 7,445 nucleotide length. The isolate was designated SCCN04 and its sequence was submitted to the GenBank database (https://www.ncbi.nlm.nih.gov/genbank) under the accession number MW178244. The phylogenetic trees' horizontal branch lengths are scaled to the number of nucleotide substitutions per site, and the trees are midpoint rooted. The percentage of trees in which the associated taxa clustered together was determined from 1,000 bootstrap replicates and is shown next to the branches (only bootstrap values ≥70 are shown). The red triangle indicates the GI.2 strain isolated in the present study.

Fig. 5. Maximum-likelihood phylogenetic trees. A – tree for the whole genome, nucleotide substitution model GTR + G + I; B – tree for the non-structural genes: nucleotides 1–5295, nucleotide substitution model GTR + G + I; C – tree for the structural genes (*VP60+VP10*): nucleotides 5296–7369, nucleotide substitution model GTR + G + I

Table 1. Recombination analysis of the SCCN04 isolate

Major parent	Minor parent	Breakpoints (nt)	P-value						
			RDP	GENECONV	BootScan	MaxChi	Chimaera	SiScan	3Seq
MT586027.1[a]	MN90145.1[a]	2858and 5137	-	-	-	2.046×10^{-7}	2.481×10^{-6}	-	4.093×10^{-5}

a – GenBank accession number; - – recombination event without significance. The P-value cut-off was set at 0.01

The nucleotide similarities of the complete genomes of 60 isolates from all over the world were compared. The similarity between SCCN04 and each isolate of those 60 ranged from 87.45% to 98.85%. The similarity among the MT586027.1 subset (the Chinese SC2020/0401 isolate submitted in 2020) was 98.85%. The whole-genome phylogenetic tree analysis results are presented in Fig. 5. Analysis of the phylogenetic tree relating the nucleotide sequences of the whole genome showed that the SCCN04 isolate formed a small branch with the SC2020/0401 isolate (MT586027.1) from China, and the relationship between these two was the closest. The isolate obtained in this research is closely related to European GI.2 isolates on a large branch.

Recombination analysis was made with seven methods in the RDP5 application: RDP, GENECONV, BootScan, MaxChi, Chimaera, SiScan and 3Seq. At least three different methods confirmed the recombination event that indicates that the 2020 isolate MT586027.1 (SC2020/0401) is the major parental virus and MN901451.1 (Bremerhaven-17) of the same year is the minor parent. The recombination breakpoints of the sequence are nt 2858 and nt 5137 ($P < 0.001$) (Table 1).

Discussion

This study performed necropsies and RT-PCR analysis on rabbits that had died on a large-scale rabbit farm in Sichuan Province, China and were suspected of being infected with RHDV GI.2. The diagnosis of GI.2 infection was confirmed. There were a total of 2,500 rabbits on the farm and 1,029 were infected and had died. The remaining rabbits were all sacrificed humanely. The early GI.2 isolates exhibited weaker virulence than the classic GI.1 (12), and the clinical manifestations were mainly subacute and chronic infections. However, recent studies (4, 6) have found that GI.2 infections are mostly acutely fatal.

Phylogenetic tree analysis was carried out based on the complete gene sequences of the isolated SCCN04 and 60 reference strains employing the nucleotide substitution model GTR + G + I, and it was found that SCCN04 had the highest consistency with the Chinese MT586027.1(SC2020/0401, 2020) strain, which was 98.85%. Among foreign isolates, the isolate had the highest consistency with the German MN901451.1 (Bremerhaven-17, 2020) strain at 98.36%. The isolates SCCN04 and SC2020/0401 were collected at the same location at the same time period, and it was to be expected that they clustered together in terms of the whole genome, the non-structural genes and the structural genes. Twenty-eight GI.2 strains including the SCCN04 isolate sequenced in this study and KM878681 (RHDV-N11, 2011) and MT586027.1 (SC2020/0401, 2020) were clustered in a large branch, indicating that SCCN04 was GI.2. The geographical location of the first isolation of 21 of the 28 (84%) GI.2 strains referenced is Europe, and the SCCN04 and LR899145.1 (GER-BB/EI16-2.L03579/2019, 2020) isolates are the closest relatives to the European strains on the evolutionary tree. Considering that the Chinese meat rabbit breeding line mainly relies on imports, and the exporting countries are mainly located in Europe, it may be inferred that GI.2 might have been introduced into China by purchasing breeding rabbits from European countries.

The isolate reported was obtained from a single rabbit. Its entire genome was amplified and sequenced, and after splicing, a full-length gene sequence of 7,445 bp was successfully obtained. Compared with the MN901451.1 strain (Bremerhaven-17), the isolated strain SCCN04 exhibited 60 base mutations in the entire gene sequence, 21 of which were missense mutations that occurred in the encoded protein p16, p23, 2C-like, and 3C-like, VPg and RdRp regions, and regions encoding the VP60 and VP10 structural proteins. Compared with the reference strain, p16 exhibited the most missense mutations among the non-structural proteins. Although the role of p16 is not clearly understood, other studies proposed a relationship between mutations in p16 and virulence (22). At the same time, the mutation of each amino acid in any protein in the SCCN04 strain might be related to variations in strain virulence. This experimental study has increased our knowledge of the molecular epidemiology of GI.2. These results also lay the foundation for future research on GI.2 virulence and pathogenesis, as well as vaccine development.

In conclusion, this study describes a rabbit haemorrhagic disease outbreak with a GI.2 genotype virus on a rabbit farm in China. Genome sequences and phylogenetic analyses indicated that the Chinese strains are closely related to the strains circulating in Europe. This analysis is critical to understanding the introduction of GI.2 into China.

* These authors contributed equally to this work.

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Animal Rights Statement: The tissue samples analysed in this study were collected from clinically diseased rabbits with the approval of the local centre of animal disease control, and animal experiments were not conducted, thus an ethical statement is not applicable.

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