Detection of a new emerging rabbit hemorrhagic disease type 2 virus (GI.2) in China

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Abstract

In May 2020, the first outbreak of rabbit viral hemorrhagic disease (RHD) caused by rabbit hemorrhagic disease virus type 2 (GI.2) occurred in Sichuan, China. The acute onset and short disease course resulted in rabbit mortalities as high as 42.86%. Currently, basic research on the etiology and genetic characteristics of GI.2 is lacking in China. Pathological changes in various tissues from infected rabbits were investigated, and the viral genome was characterized. This study used RT-PCR, histopathology, scanning electron microscopy, and whole genome sequencing analysis to identify the pathogen in samples from infected rabbits that had died. RT-PCR identified the presence of GI.2. The histopathology revealed liver cell necrosis and hemorrhaging into lung alveoli. Electron microscopy demonstrated spherical GI.2 particles that were 40 nm in diameter with an isometric interior. The gene sequence length of the isolate was 7,464 bp and termed GI.2/ CN/SC-04. The phylogenetic analysis of the GI.2 genotype showed that the strain from GI.2/CN/SC-04 clustered into a separate group compared to the isolate from China (MT586027.1). Phylogenetic analysis based on whole sequences and showed that GI.2/CN/SC-04 strains were identical and clustered with other strains of GI.2 circulating globally. The results of recombination analysis showed that GI.2/CN/SC-04 was recombined from MT586027.1 strain (main parent strain) and MN90145.1(secondary parent strain), and both the two recombination breakpoints 2858-5137nt. This was the study to report that GI.2 had been isolated in China, to characterise the full genome sequence and provide phylogenetic insights into the origin of this

exotic incursion. development.

Keywords: China; rabbit hemorrhagic disease type 2 virus; whole genome sequence; genetic variation

1. Introduction

Rabbit hemorrhagic disease (RHD), also known as rabbit plague, and rabbit hemorrhagic pneumonia, is caused by the rabbit hemorrhagic disease virus (GI.1), which belongs to the family Caliciviridae (*Lagovirus*). RHD is an acute, septic, highly lethal, infectious disease in rabbits. The disease caused by GI.1 first broke out in Wuxi, Jiangsu, and other locations in China in 1984[Liu et al., 1984]. Young rabbits older than two months of age and adult rabbits are susceptible to classic GI.1, with an incidence rate of 100% and a fatality rate of 90%. Indeed, while GI.1 exclusively kills adults, GI.2 also lethally affects sub-adults and kittens as young as 11 days (Dalton et al., 2012). Infected rabbits that die exhibit hemorrhage and enlargement of the liver, spleen, kidney, and other organs, as well as congestion in the nasal cavity and trachea [Abrantes et al., 2012; Michael et al., 2007]. Rabbit hemorrhagic disease virus type 2 (GI.2) is a novel lagovirus, and also a variant strain of GI.1. Molecular epidemiological studies have demonstrated that GI.2 has replaced GI.1 as the current epidemic strain in France, Spain, Portugal, Australia and others. [Dalton et al., 2014; Mahar et al., 2018]. Under natural conditions, GI.2 infects rabbits and hares, but rabbits are more susceptible, especially young and juvenile rabbits. Water and food contaminated with GI.2 allow entry into the host through the respiratory and digestive tracts due to epithelial cell HBGA attachment factors [Nyström et al., 2011]. GI.2 infection results in hepatic necrosis and diffuse intravascular coagulation, leading to substantial organ bleeding [Neimanis et al., 2018].

GI.2 has no envelope structure. The virus is spherical with a diameter of 32-44nm and displays icosahedral symmetry. The virus is a single-stranded, positive-stranded RNA virus with a full-length genome of 7,442bp. The virus is different from the GI.1 and GI.1a gene sequences [Meyers et al., 2000; Parra et al., 1990; Lopes et al., 2015], which nucleotide identity display 82.4% in the genome structure of GI.2 and GI.1 [Le Gall-Reculé et al., 2013]. The GI.2 genome has two open reading frames, ORF1 and ORF2. The ORF1 encodes a polyprotein, which is subdivided into multiple non-structural proteins, including p16, p23, p29, 2C-like protein, 3C-like protein, VPg, RNA-dependent RNA polymerase (RdRp), and structural protein VP60[Dalton et al., 2014; Meyers et al., 2000; Knig et al., 1998]. VP10 is a minor structural protein encoded by the 3'-end gRNA and sgRNA in ORF2. Some studies have indicated that VP10 can increase the level of virus replication and promote cell apoptosis [Liu et al., 2008]. Also, other studies have confirmed that VP10 could down-regulate the expression of VP60[Chen et al., 2009]. Therefore, this suggested that mutations in VP10 might affect host cell performance, virus replication, and virion release.

In April 2020, GI.2 broke out for the first time in China[Hu et al., 2020]. According to the GI.2 full gene sequence registered by NCBI (GenBank accession number: MN901451.1), complete the gene sequence and compare it with the registered classic GI.1, GI.2, GI.3 and other

sequences. After analysis of the results, a phylogenetic tree was constructed to clarify the evolutionary history of the isolates, enrich the GI.2 gene sequence materials, and provide references for GI.2 diagnosis, GI.2 vaccine research and development, and research into disease mechanisms to allow the development of new treatments.

2. Materials and methods

2.1 Sample collection and viral nucleic acid extraction

In April 2020, we collected 100 dead rabbits (20 to 60 days old) from a rabbit farm in Jintang City, Sichuan Province, China, suspected of being infected with GI.2. Under strict biosafety conditions, the dead rabbits were dissected, and the lesions were observed. Visceral tissues, including the 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 pasteurella rabbits, European brown hare syndrome virus (EBHSV), rabbit hemorrhagic disease virus (RHDV) at the Sichuan Agricultural University (Chengdu, Sichuan).

Ther tissue samples of liver, lung, heartand spleen were homogenized, diluted tenfold with phosphate-buffered saline (PBS; 0.1 M, pH 7.4), and stored at -70°C until analyzed. The samples were frozen and thawed three times to release the virus, centrifuged at 8,000 rpm for 10 minutes at 4°C, and DNA and RNA were collected from the supernatant. The viral genomic RNA was extracted using a total viral RNA extraction kit (TaKaRa, China) and reverse transcribed into first-strand cDNA using M-MLV reverse transcriptase (TaKaRa). The cDNA was held at -20°C for later use.

2.2 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 established previously for RT-PCR carried out in our laboratory [Yang et al.,2016] (Forward 5'-GGGTGTCATATCCACCCCAAA-3'; Reverse primer 5'-CCCAGGTTGAACACGAG-3'). The target fragment was 441bp. After the reaction, the PCR products were detected using 1% agarose gel electrophoresis. The amplicons were gel purified, cloned, and recombinant plasmid of each clone were sequenced employing Sangon Biotech Shanghai Co., Ltd. The recovered target bands were sent to for sequencing. The sequencing results were compared and analyzed by BLAST.

2.3 Transmission electron microscopy (TEM)

A liver tissue sample from an infected rabbit was homogenized in PBS, frozen and thawed three times to release the viral particles, then centrifuged at 8,000 rcf/ for 5 minutes. The resulting supernatant was used to create a virus solution. NaCl was added to the virus solution to produce a final concentration of 0.5 mol/L to precipitate the viral particles. An equal amount of 6% PEG 6000 was added, and the solution was held at 4°C overnight. The solution was centrifuged at 12,000 rcf /min for 1h, the virus pellet was collected, and the supernatant was discarded. After adding an appropriate volume of PBS, the solution was centrifuged for 1 hour. The resulting pellet was washed twice and held at 4°C. Subsequently, the sample was sent to Wuhan servicebio

technology CO., LTD for analysis using TEM.

2.4 GI.2 determination and sequencing of the full-length genome

The DNA library was sent to Shanghai Personalbio Biotechnology Co., Ltd. for high-throughput sequencing using Illumina NovaSeq, using Bremerhaven-17(MN901451.1) as the reference genome. First, A5-MiSeq (Coil D et al., 2014) and SPAdes (BankevichA et al., 2012) were used to de-assemble 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 was the same the blastn (Altschul SF et al., 1990) alignment was performed on the NT library on the NCBI, and the viral genome sequence of each splicing result was selected. Integration of splicing results: use MUMmer (KurtzS et al., 2004) software to perform collinearity analysis to determine the positional relationship between contigs, and fill gaps between contigs; use Pilon (Walker BJ et al., 2014) The software corrects the results to obtain the final viral genome sequence.

2.5 Genome sequence analysis

For phylogenetic analysis, the best-fit model of nucleotide substitution was assessed with MEGA X under the Akaike Information Criterion (AIC) [Tamura et al.,2016]. 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 4.40, 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.

3. Results

3.1 Visceral lesions and nucleic acid test results obtained from sick and dead rabbits

During the necropsy of the sick and dead rabbits, areas of hemorrhage were seen in the lungs (Figure 1A) and epicardium. Resection of the tracheas revealed considerable hemorrhage, with the presence of some foam (Figure 1B). The livers were enlarged, friable, and also exhibited hemorrhage (Figure 1C). Most of the feces samples were dry. The sick rabbits had urine in their bladders, and some presented with oral and nose hemorrhaging (Figure 1D).

3.5 Histopathology

All animals displayed acute necrosis or apoptosis of individual hepatocytes that was characterized as severe, panlobular necrotizing hepatitis with massive congestion and acute hemorrhage (Figure 2c-d). Some lesions occurred in the alveoli. Some alveolar spaces were narrowed and exhibited mild hemorrhage, while other alveolar spaces were completely filled (Figure 2b). Splenic lymphocytes were necrotic, and there was hemorrhage and congestion in the red pulp (Figure 2a). There were no apparent abnormalities observed in the heart or kidneys.

3.4 Transmission electron microscopy (TEM) results

Examination of the GI.2 virus sample using TEM revealed a clear background with no viral

envelope present. The virus particles were 40 nm in diameter and presented an icosahedral symmetric structure(Figure 3).

3.5 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 (Figure 4). After the product was sequenced, it was analyzed by BLAST, which indicated that the target fragment gene sequence was in GenBank. The sequence match for the GI.2 VP60 gene (accession number: MT586027.1) was 98.63%.

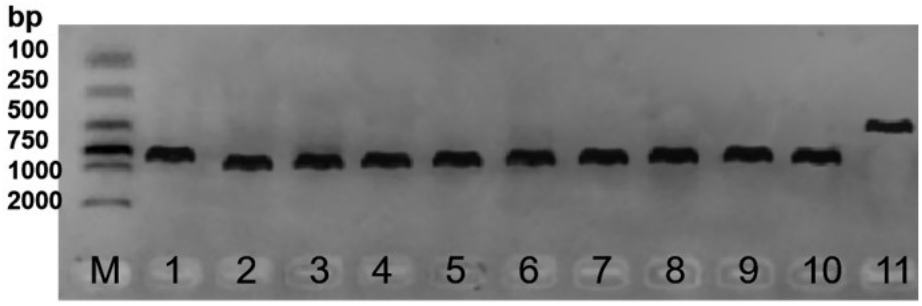
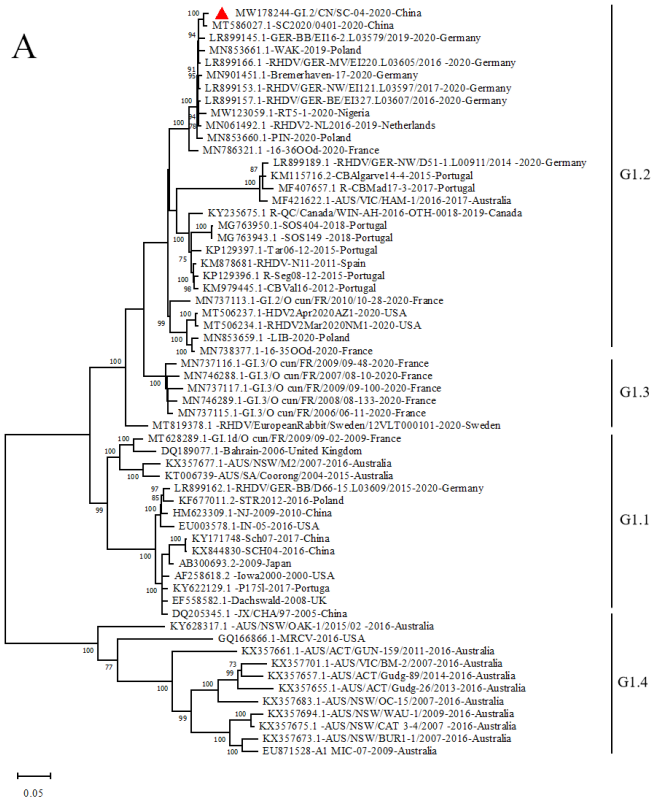


Figure 4. PCR amplification results of the whole gene from strain GI.2/CN/SC-04.

M: DNA Marker DL2000；1~11: GI.2/CN/SC-04 A ~ K gene fragment amplification target band

The genomes of GI.2 were amplified directly from tissue homogenates and sequencing results showed 7445 nucleotide length of the genomes. The sequences were submitted to the publicly available GenBank database (https://www.ncbi.nlm.nih.gov/genbank) under the accession numbers MW178244.



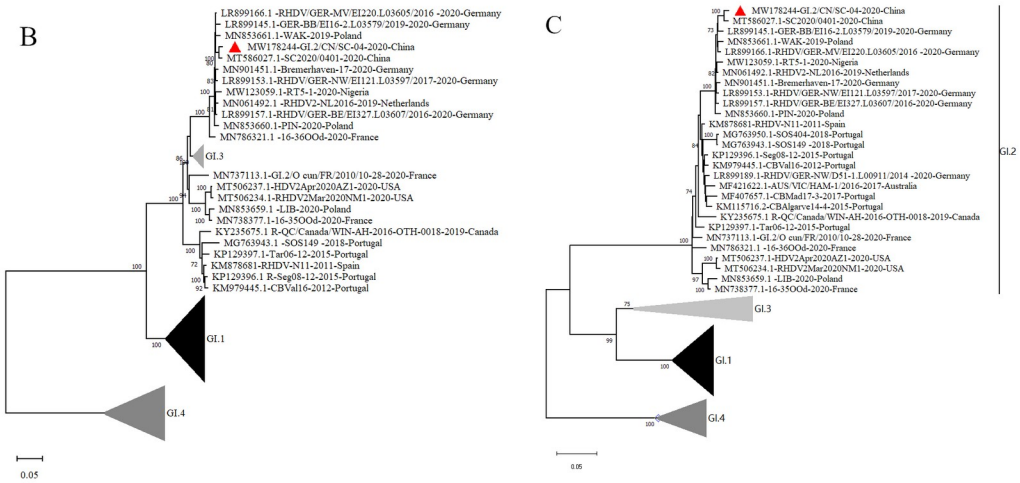


Figure 5. Maximum likelihood (ML) phylogenetic trees for (A)the_whole genome; nucleotide substitution model GTR+G+I, (B) the nonstructural genes; nucleotides 1–5295; nucleotide substitution model GTR+G+I and (C) the structural genes(VP60+VP10); nucleotides 5296–7369; nucleotide substitution model GTR+G+I. Horizontal branch lengths are drawn to scale 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 1000 bootstrap replicates and is shown next to the branches (only bootstrap values ≥ 70 are shown). The black triangle indicates the GI.2 strain found in the present study.

The nucleotide similarities of the complete genomes of 60 isolates from all over the world were compared. The similarity between GI.2/CN/SC-04 and the 60 isolates ranged from 87.45% to 98.85%. The similarity among the MT586027.1 subset was 98.85%._The whole genome phylogenetic tree analysis results are seen in Figure 5. The analysis of a phylogenetic tree constructed based on the nucleotide sequence of the whole genome showed that the GI.2/CN/SC-04 isolate formed a small branch with the MT586027.1 isolate from China, and the relationship was closest. It is closely related to European GI.2 isolates on a large branch.

Comparison with the nucleotide sequence of the German isolate MN901451.1, the nucleotide sequence of GI.2/CN/SC-04 exhibited many base changes. Combined with the amino acid codon comparison table, numerous missense mutations were observed in the region encoding the p16 protein. There were four mutations between amino acid residues 1-143, including Leu55→Phe55, Glu57→Asp57, Lys134→Glu134, and Asn138→Ser138. The region encoding the p23 protein between amino acid residues 144-367 exhibited two mutations, including Ala284→Thr284 and Thr337→Ile371. There were three mutations in the region encoding the 2C-like protein between amino acid residues 368-718, including Ile423→Val423, Ala637→Val637, and His644→Ser644. The region that encoded p29 exhibited one mutation, which was Lys768→Arg768. The region encoding VPg protein between 995-1108 amino acid residues exhibited one mutation, Asn1105→lys1105. Three mutations were observed in the region encoding RNA-dependent RNA polymerase (RdRp) between 1279-1750 amino acid residues, including Ser1617→arg1616, Lys1680→Asn1680, and Ala1731→glu1730. One mutation was found in the region encoding the structural protein VP60, Phe1885→Ser1885. Six mutations were discovered in the coding structural protein VP10 that included Trp2348→Arg2348, Arg2373→Gly2373,

Phe2387→Leu2387, Ser2406→Gly2406, His2424→Tyr2424, and Val2434→Ile2434. It remains to be determined whether these amino acid differences are critical for GI.2 infection.

Recombination analysis was carried out by RDP4 software, and the results showed that after 7 methods of RDP, GENECONV, BootScan, MaxChi, Chimaera, SiScan and 3Seq, it was confirmed that MT586027.1 is the major parental virus, and MN90145.1 is the minor parent. Its recombination breakpoints are nt 2858 and nt 5137 ($P<0.001$) (Table 1).

4. Discussion

This study performed necropsies and RT-PCR analysis on rabbits that had died and were suspected of being infected with GI.2 at a large-scale rabbit farm in Sichuan Province, China. The diagnosis of GI.2 infection was confirmed. There were a total of 2500 rabbits on the farm, and 1029 were infected and died. The remaining rabbits were all killed and disposed harmlessly. The farm was introduced for the last time in August 2019. Based on the time of introduction and the longest incubation period of the disease, it is very unlikely that the introduction will cause the disease. Due to the impact of the new crown epidemic, the farm has returnees from countries where GI.2 is endemic, and it is not ruled out that the possibility of foreign introduction through personnel or article contamination etc.

The early GI.2 isolates exhibited weaker virulence than the classic GI.1[Le et al., 2013], and the clinical manifestations were mainly subacute and chronic infections. However, recent studies[Capucci et al., 2017; Dalton et al., 2012] have found that GI.2 infections are mostly acutely fatal, and the infections primarily occur in young and juvenile rabbits. In this study, lesions were observed at necropsy in dead rabbits that had been collected from a rabbit farm in Chengdu, Sichuan Province, where a GI.2 outbreak was suspected. The visceral tissues from dead rabbits were collected and determined to be GI.2 positive by RT-PCR. Animal regression experiments demonstrated that the pathological changes of rabbits regression with GI.2 included hemorrhages in the lungs and epicardium as well as hepatic enlargement, friability, and hemorrhage. These results are consistent with other studies that reported on the clinical symptoms observed in rabbits infected with GI.2.

A GI.2 isolate, GI.2/CN/SC-04, was obtained from a single rabbit, and the entire genome of that isolate was amplified and sequenced. After splicing, a full-length gene sequence of 7,464 bp was successfully obtained. Compared with the MN901451.1, the isolated strain, GI.2/CN/SC-04, exhibited 60 base mutations in the entire gene sequence, 11 of which were missense mutations that occurred in the encoded proteins p16, p23, 2C-like, and 3C-like, VPg, RdRp regions, and regions encoding structural proteins VP60 and VP10. Compared with the reference strain, p16 amino acids exhibited the most missense mutations among the non-structural proteins. Although the role of p16 is not clearly understood, other studies have proposed a relationship between mutations in p16 and virulence [Silvério et al., 2018]. At the same time, the mutation of each protein amino acid in the GI.2/CN/SC-04 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 into GI.2 virulence and pathogenesis as well as vaccine development.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

ETHICAL STATEMENT

The tissue samples analysed in this study were collected from clinical diseased rabbit with the approval of local centre of animal disease control, and animal experiments were not conducted, thus the Ethical Statement is not applicable.

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