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Identification and molecular characterization of a rabbit hemorrhagic disease virus variant (KV0801) isolated in Korea

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Abstract : Rabbit hemorrhagic disease (RHD) is caused by RHD virus (RHDV) and is one of the most fatal diseases of rabbits. Acute death of rabbits occurred in a farm located in the Gyeonggi province of South Korea. The virus was isolated and confirmed as RHDV based on reverse transcription polymerase chain reaction and hemagglutination assay (HA), and the isolate was designated as KV0801. The nucleotide sequence of the complete VP60 gene of KV0801 was determined and the corresponding amino acid sequence was deduced. Molecular analysis showed that the KV0801 isolate can be classified as a pandemic antigenic variant strain, RHDVa. The VP60 nucleotide sequence and deduced amino acid homology between KV0801 and other Korean isolate, RHF89, which was isolated in 1988, were 92.1 and 94.3%, respectively. The pathogenicity of the KV0801 isolate at an HA titer ranging from 16,384 to 0.16 HA units was evaluated in five-month-old SFP rabbits. The rabbits inoculated with KV0801 isolate containing more than 1.63 HA units died within six days of inoculation. These results suggest that a highly pathogenic RHDVa is circulating in the rabbit populations of Korea.

Keywords : characterization, isolation, Korea, rabbit, RHDV

Introduction

Approximately 302,000 rabbits are raised on 8,000 farms throughout South Korea at the present time [13]. These rabbits are mainly raised for their meat (as a food source) and for clothing (i.e., fur and leather), although their market is not as big in Korea as that of other domesticated animals. Rabbit hemorrhagic disease (RHD) is one of the most fatal diseases affecting the rabbit industry and is caused by RHD virus (RHDV). RHDV is a member of the family *Caliciviridae*, genus *Lagovirus*, and 7.4 kb of its genome encodes a 257 kDa polyprotein. Posttranslational processing within this polyprotein results in several mature non-structural proteins, which include helicase, protease, RNA dependent RNA polymerase, the 60 kDa major capsid protein (VP60) and minor structural proteins [12, 23]. As with other caliciviruses, RHDV has an icosahedral shape and is about 30 nm in diameter. Based on genetic and antigenic analyses, the RHDV species was divided into at least two subtypes [2, 20]. Although antigenic variants have been reported, only one serotype has been identified to date [7, 11].

After the original identification of RHDV in Chinese rabbits (*Oryctolagus cuniculus*) in 1984, the disease has been reported in several other countries, which include Italy, France, the USA and Korea; thus, the disease is endemic in East Asia and Europe [6, 9, 11, 17]. The cause of death by RHD in rabbits is related to hepatocellular necrosis and disseminated intravascular coagulation [16, 21]. Rabbit hemorrhagic disease is mainly transmitted by close contact with infected rabbits and contaminated fur. Rabbits infected with RHDV develop fever rapidly, and sudden death occurs within 12 to 36 h after natural exposure, in the acute form. In some cases, rabbits exhibit a blood-tinged foamy nasal discharge near death, as well as hemorrhagic lesions in several organs, which include the liver and lungs. RHDV should be differentiated from European brown hare syndrome virus (EBHSV)

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and rabbit calicivirus (RCV). Rabbits infected with EBHSV exhibit clinical signs similar to those associated with RHDV infection; in contrast, rabbits infected with RCV do not show any clinical signs.

Several methods, which include electron microscopy, immunostaining, Western blotting, hemagglutination assay (HA) and reverse transcription polymerase chain reaction (RT-PCR), have been used for the diagnosis of RHD in infected samples [2, 16, 23, 24]. RHDV infection can also be diagnosed through the detection of a specific antibody response using the hemorrhagic inhibition test or comparative ELISA [18, 19].

Recently, a case of RHD occurred in a farm located in the Gyeonggi province of South Korea. In this study, we investigated the molecular characterization and the pathogenicity of the RHDV Korean isolate (KV0801) obtained in 2008.

Materials and Methods

Samples

Two isolates, designated as KV0801 and RHF89, were used for the diagnosis, molecular analysis of the VP60 gene and pathogenicity assessment. KV0801 was originally isolated from the liver of a rabbit in 2008. The isolate was passaged once in an RHDV seronegative New Zealand rabbit. The liver of the inoculated rabbit was homogenized in 10% phosphate buffered saline (PBS, pH 7.2). The RHF89 isolate, which was isolated in 1988 [1], was incorporated into this study. In order to isolate the RHDV, rabbit tissue samples, i.e., rabbit livers and feces, were obtained from a Korean farm that had recently experienced an RHD outbreak and from two adjacent farms that had no history of RHD.

HA test

An HA test was carried out according to the standard method described in the office international des epizooties manual [15]. In short, 50 μL of homogenized liver (in 10% PBS) was diluted with PBS, and 50 μL of 0.75% human type O erythrocytes was added. After incubation at 4°C for 1 h, the HA titer was expressed as the reciprocal of the highest dilution of antigen showing hemagglutination.

RNA extraction and RT-PCR

Viral RNA was extracted from liver samples using an RNA extraction kit (Bioneer, Korea), according to the manufacturer's instructions. RT-PCR was carried out for the detection of RHDV-genomic sequences using specific primers (RHDVF, RHDVR, RHDV1F, RHDV1R, RHDV2F and RDHV2R) that amplify a VP60 region of RHDV and are listed in Table 1. The RT-PCR was performed in a reaction mixture containing 20 μL of denatured RNA, 1 μL of each primer (50 pmol), 10 μL of 5 × buffer (12.5 mM $MgCl_2$), 2 μL of enzyme mix (reverse transcriptase and *Taq* polymerase) and 14 μL of distilled water (Qiagen, Germany). The

Table 1. List of the oligonucleotide primers used for reverse transcription polymerase chain reaction of rabbit hemorrhagic disease virus (RHDV)

Target virus	Primer designation	Oligonucleotide sequence (5' to 3')	Size of amplicon
Rabbit calicivirus	RCVF	ATG GCT ACT ACT CAT ACG CTT CTG	818 bp
	RCVR	GGG TTC AAC CCC AGG CGG CA	
European brown hare syndrome virus	EBHSF	CCG TCC AR*C ATT CGT CCT GTC AC	265 bp
	EBHSR	CAT CAC CAG TCC TCC GCA CCA C	
RHDV	RHDVDF	GAC TAC TCA AAG TGG GAC TCC	340 bp
	RHDVDR	TCG GAG TCA TGG CAT ACA CG	
	RHDV1F	TGT TAT GGA GG CAA AGC CCG T	930 bp
	RHDV1R	GGA AAA CCC CCC AGG TAC TGG TTG	
	RHDV2F	GCA ACC AGT ACC TGG AGG GT	950 bp
	RHDV2R	TTA TCA GAC ATA AGA AAA GCC	

*R = A/G.

cycling profile was as follows: cDNA synthesis at 42°C for 30 min, followed by 35 cycles of denaturation at 95°C for 30 sec, annealing at 50°C for 30 sec, extension at 72°C for 1 min and a final extension at 72°C for 5 min. RT-PCR products were visualized via electrophoresis on a 1.5% agarose gel containing ethidium bromide. The PCR products were purified using a gel extraction kit (Bioneer, Korea) and were subsequently ligated into the pGEM-T easy vector (Promega, USA). Plasmid DNA was isolated from amplified *Escherichia coli*, and recombinant plasmids were identified using *Eco*RI enzyme digestion (Bioneer, Korea) and DNA sequencing.

DNA sequencing and phylogenetic analysis

Sequencing analysis of the purified plasmids was performed using an MJ Research PTC-225 Peltier Thermal Cycler and ABI PRISM BigDye Terminator Cycle Sequencing kits with AmpliTaq DNA polymerase (FS enzyme) (Applied Biosystems, USA), according to the protocols supplied by the manufacturer. Single-pass sequencing was performed for each template using universal primers (e.g., SP6 and T7). The fluorescent-labeled fragments were purified from the unincorporated terminators using an ethanol precipitation protocol. The samples were resuspended in distilled water and subjected to electrophoresis in an ABI 3730xl sequencer (Applied Biosystems, USA). Both DNA strands were sequenced to verify the sequences. Sequences used for phylogenetic analysis in this study were obtained form the GenBank database and their accession number (strain) were as follows: EF55852 (Dachwald), AF25818 (Iowa2000), AJ303106 (00Reu), DQ205345 (JxCHA97), AF453761 (TR), AJ302016 (99-05), AJ969628 (03-24), DQ069280 (whn/China/01/2005), DQ069281 (whn/China/02/2005), DQ069283 (whn/China/03/2005), DQ 280493 (WHNRH), AY523410 (CD/China), DQ841708 (CUB5-05), AY269825 (NJ/China/1985), M67473 (FRG), AF231353 (Newzealand), AF402614 (WX/China/1984), U49726 (Haute-sanoe), AF295785 (Mexico89), EU003580 (Korea90), X87607 (BS89), AJ0006019 (Rainham), EF558585 (Hegenow), AY926883 (Ireland18), EF558577 (Meiningen), Z24757 (AST89). Phylogenetic trees were obtained, and homology analyses were carried out using the DNASTAR software (DNASTAR, USA) and the DNASIS software (Hitachi Software, Japan), respectively.

Animal experiments

To evaluate the pathogenicity of the KV0801 isolate, 24 RHDV seronegative SPF rabbits aged five months were divided into six groups (four rabbits per group) and were inoculated by an intramuscular route with 1 ml of the isolate at a titer ranging from 16,284 to 0.16 HA units. Among them, four rabbits were intramuscularly inoculated with saline only and were considered as the negative control group. The clinical signs of the rabbits were monitored for 10 days.

Results

Identification of RHDV

Disease associated with continuous and fatal death was reported in 4 to 7-week-old rabbits raised on farms in the Gyeonggi province. RT-PCR analysis using specific primers for RHDV, EBHSV and RCV was performed in rabbit liver homogenates to diagnose the cause of death. In addition, samples obtained from two adjacent farms were also subjected to the agent identification procedure described above. The procedure was intended to determine whether any causative agents were transmitted to the adjacent farms. Amplified gene products corresponding to RHDV were detected on a 1.5% agarose gel (Fig. 1). The propagation of the causative agent was attempted in Vero and TF104 cells

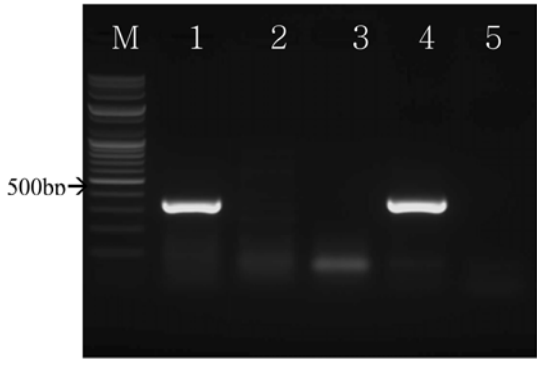

Fig. 1. Detection of the VP60 gene of rabbit hemorrhagic disease virus (RHDV) using reverse transcription polymerase chain reaction (RT-PCR) and electrophoresis on a 1.5% agarose gel. The expected amplicon size was 430 bp. M: 100 bp DNA ladder; lanes 1-3: KV0801 isolate; lane 4: RHF89 isolate; lane 5: negative control. To distinguish RHDV from European brown hare syndrome (EBHS) and rabbit calicivirus (RCV), RT-PCR of samples resolved in lanes 2 and 3 was carried out using EBHS virus- and RCV-specific primers, respectively.

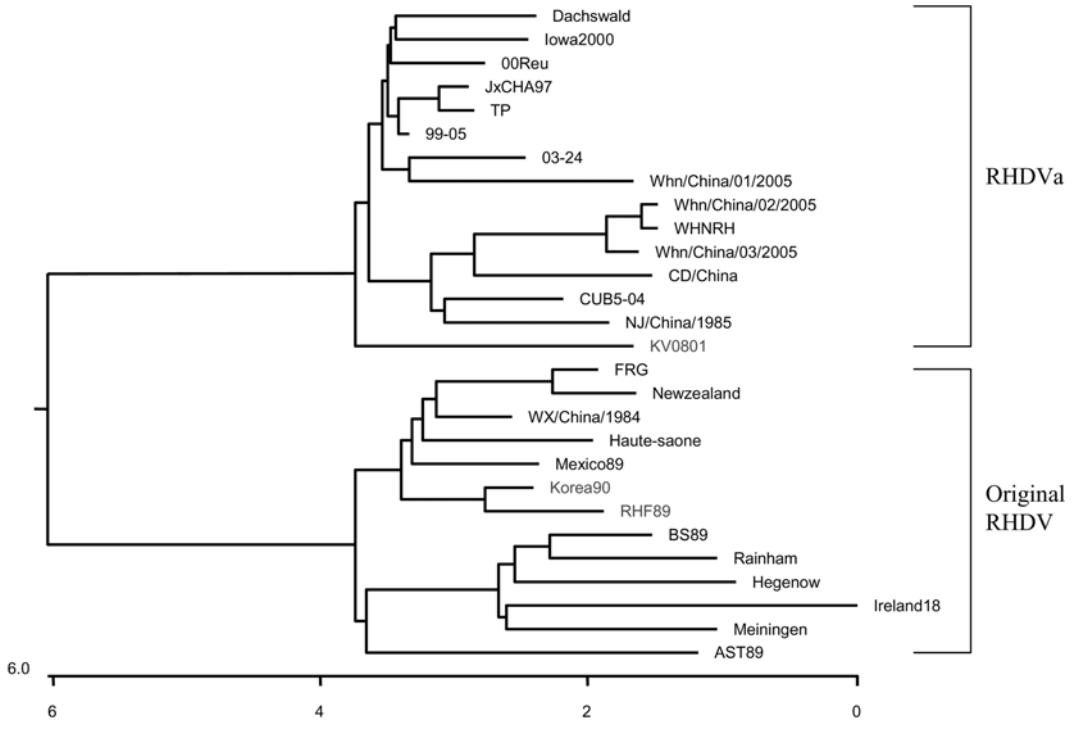

Fig. 2. Phylogenetic tree of the VP60 gene of the 28 rabbit hemorrhagic disease viruses. The tree was generated from the comparative alignment of complete sequences from the VP60 gene of RHDVs.

using the homogenated livers; however, we were unable to cultivate any viruses in these cells.

Sequence analysis of RHDVs

The gene encoding the VP60 protein from the KV0801 and RHF89 isolates was amplified into two fragments using primer sets designated as RHDV1 and 2, which were cloned into the pGEM-T easy vector and sequenced. The complete VP60 gene sequence data for KV0801 and RHF89 were deposited in GenBank (NCBI), with accession numbers FJ212322 and FJ212323, respectively. The VP60 gene sequences of all 26 viruses obtained from GenBank were compared with those of the Korean isolates to determine whether the Korean isolates were related to the pandemic RHDVa. Based on nucleotide sequence analyses of the KV0801 isolate and reference strains, the KV0801 and RHF89 isolates were classified as a new RHDVa subtype and as the original RHDV subtype, respectively (Fig. 2). The highest sequence homology of the KV0801 isolate corresponded to the JX/CHA/97 and TP strains of RHDVa (97.5 and 97.4%, at the nucleotide level, and 99.1 and 98.9%, at the amino acid level, respectively). The nucleotide and deduced amino acid homology of the VP60 gene between the KV0801 and RHF89 isolates were 92.1 and 94.3%, respectively. An RHDVa-specific antigenic epitope was predicted in the hypervariable region of VP60 (amino acids 344-370) [2]. The amino acid alignment of the three Korean isolates showed that eight residues in the antigenic epitope region were substituted among the three isolates (Fig. 3).

Pathogenicity of the KV0801 isolate in rabbit

The pathogenicity of the KV0801 isolate at a virus titer ranging from 16,384 to 0.16 HA units was evaluated in rabbits via intramuscular inoculation. Rabbits inoculated with KV0801 isolate containing more than 1.63 HA units died within six days of inoculation (Fig. 4). None of the control rabbits died during the experiment. Most of the rabbits inoculated with over 1.63 HA units developed anorexia 12 h post inoculation, and several rabbits developed a blood-tinged foamy nasal discharge and convulsion near

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Korea90   1 MEGKARAAPQGEAAGTATTASVPGTTTDGMDPGVVATTSVITAENSSASIATAGIGGPPQ  60
RHF89       ..........................................................
KV0801      ......T.....T.....................A...V........V..........

Korea90  61 QVDQQETWRTNFYYNDVFTWSVADAPGSILYTVQHSPQNNPFTAVLSQMYAGWAGGMQFR 120
RHF89       ..........................................................
KV0801      ..........................................................

Korea90 121 FIVAGSGVFGGRLVAAVIPPGIEIGPGLEVRQFPHVVIDARSLEPVTITMPDLRPNMYHP 180
RHF89       ..........................................................C..
KV0801      ..........................................................

Korea90 181 TGDPGLVPTLVLSVYNNLINPFGGSTSAIQVTVETRPSEDFEFVMIRAPSSKTVDSISPA 240
RHF89       ..........................................................
KV0801      ..........................N.........D.................V...

Korea90 241 GLLTTPVLTGVGNDNRWNGQIVGLQPVPGGFSTCNRHWNLNGSTYGWSSPRFADIDHRRG 300
RHF89       ......................E...........................................
KV0801      ..........................................................

Korea90 301 SASYSGSNATNVLQFWYANAGSAIDNPISQVAPDGFPDMSFVPFNGPGIPAAGWVGFGAI 360
RHF89       ....P.....................................Y.....T.....T.....
KV0801      ......NTS.....................................S.SI.T.....G.

Korea90 361 WNSNNGAPNVTTVQAYELGFATGAPGNLQPTTNTSGAQTVAKSIYAVVTGTTQNPAGLFV 420
RHF89       ..........................................................
KV0801      ........AA..............N.....................N...T....

Korea90 421 MASGVISTPNASAITYTPQPDRIVTTPGTPAAAPVGKNTPIMFASVVRRTGDVNATVGSA 480
RHF89       ....................H.....................W.....
KV0801      ..........N.V.....................................AA..T

Korea90 481 NGTQYGTGSQPLPVTIGLSLNNYSSALMPGQFFVWQLTFASGFMEIGLSVDGYFYAGTGA 540
RHF89       ..........................................................
KV0801      ..........................................................

Korea90 541 STTLIDLTELIDVRPVGPRPSKSTLVFNLGGTANGFSYV 579
RHF89       ...................................
KV0801      ..............................T......
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Fig. 3. Amino acid alignment of the VP60 protein from the three Korean RHDV isolates (KV0801, RHF89, Korea90). The hypervariable region was underlined. Identical amino acids were indicated by dots.

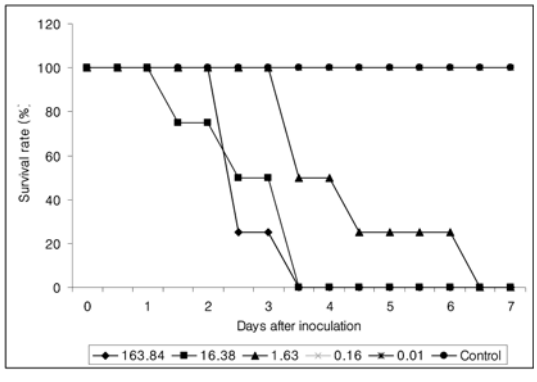

Fig. 4. Survival rate of the rabbits inoculated with the KV0801 RHDV isolate. Each group comprised four rabbits and was inoculated with the KV0801 strain at a titer ranging from 163.84 to 0.01 hemagglutination assay (HA) units.

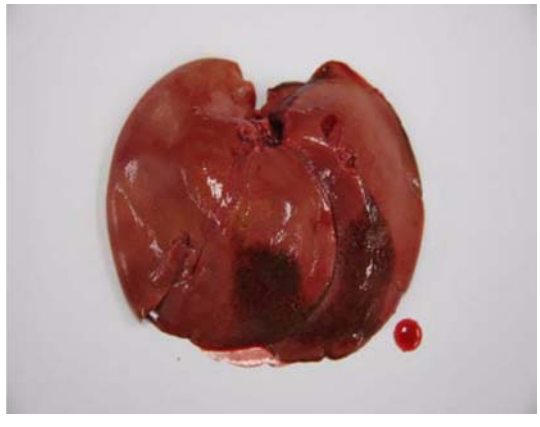

Fig. 5. Liver of a rabbit inoculated with 163.84 HA units of the KV0801 RHDV isolate. Multifocal necrosis and hemorrhage were detected in several lobes.

death. Typical RHD lesions were found at necropsy (e.g., necrosis; Fig. 5). Liver homogenates of the rabbits inoculated with the KV0801 isolate had a high HA titer, which ranged from 1,024 to 16,384 HA units.

Discussion

Since the original identification of RHDV in China in 1984, many other countries have reported outbreaks of RHD and have submitted the nucleotide sequences of the viruses to several genetic information banking systems. Although outbreaks of RHD were reported in Korea in 1988, there are few publications on the molecular epidemiology of RHDV in this country [1, 17]. Because the acute or subacute forms of the disease may also be caused by other viruses, which include EBHSV and Myxoma virus (the latter causes the systemic and fatal disease known as myxomatosis [5]), differential diagnostic methods (e.g., RT-PCR using specific primers) should be performed. In this study, RHDV was detected in only one of three Korean farms, which indicates that a close examination should have been carried out to find the cause of death in the other farms.

The genetic relationship among the various RHDV strains has been described in previous reports [3, 11, 14]. The emergence of the RHDVa strain was identified based on the molecular analysis of VP60 sequences during the 2003 outbreak of RHD in Hungary [10]; however, only one serotype of RHDV is known to date. Here, the complete nucleotide sequence and deduced amino acid sequence of the VP60 gene of the KV0801 isolate were determined and compared with those of other RHDVs. Phylogenetic analysis revealed that there were two RHDV subtypes in Koreas and that the KV0801 isolate represented a new subtype of RHDV, termed RHDVa. The earlier Korean isolates RHF89 and Korea90, which were isolated from rabbits in 1988 and 1990, were classified into the original RHDV subtype. The deduced amino acid sequence of the VP60 gene of the KV0801 isolate was aligned with those of other Korean isolates. The KV0801 isolate had 94.5 and 95.7% amino acid similarity with RHF89 and Korea90, respectively (Fig. 3). RHDV has been shown to be replaced by the RHDVa subtype in Europe and America [11]. The Korean isolate KV0801 was found to be most closely related to the JX/CHA/97 strain (97.5% identity), which was isolated in China in 1997. This suggests that this genetic feature may reflect a geographic relationship among the isolates. It is also assumed that this RHDVa subtype has been endemic since the late 1990s.

According to the experimental RHDV infection in rabbits reported in previous studies [4, 21], animals inoculated with RHDV develop sudden death within three days of inoculation. Recently, Liu *et al.* [8] reported that the full-length cDNA clones were infectious when directly injected into rabbits. In this study, rabbits inoculated with the KV0801 isolate at titer over 1.63 HA units died within 6 days of inoculation; therefore, the new Korean isolate KV0801 could be considered as virulent and was classified as a RHDVa subtype. These results may contribute to the understanding of the molecular epidemiology related with RHDV in Korea.

The recombinant inactivated RHDV vaccine termed "RabbiShot RHD Plus" is commercially available to protect rabbits from the fatal disease and has been used in Korea since the mid 1990s. Further investigation is needed to assess whether the recombinant vaccine confers sufficient protection against challenging RHDVa subtypes, which include KV0801.

Acknowledgments

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