Global Journal of Molecular Sciences 7 (1): 01-10, 2012
ISSN 1990-9241

DOI: 10.5829/idosi.gjms.2012.7.1.66132

Diagnostic Applications of Reverse Transcriptase-Polymerase Chain Reaction, Gel Electrophoresis and Western Immunoblot for Detection of Rabbit Hemorrhagic Disease Virus

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Abstract: Rabbit hemorrhagic disease virus (RHDV), a member of the family *Caliciviridae* comprising positive-stranded RNA viruses, is a highly virulent pathogen of rabbits. Until recently, studies into the molecular mechanisms of RHDV replication and pathogenesis have been hindered by the lack of an *in vitro* culture system and reverse genetics. This study described the adaptation of field isolates from rabbits infected with RHDV to growth in rabbit kidney-13 cells (RK-13) and the application of reverse transcriptase-polymerase chain reaction (RT-PCR) on culture supernatants to detect whether the genome of RHDV has been mutated and to understand the genetic relationship between the current RHDV isolates in Egypt and the foreign isolates.The viral polypeptides have also been investigated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and western immunoblot analysis. This data confirmed that the viral RNA could efficiently be replicated in RK-13cells and resulted in typical cytopathic effects after 24-72 hour.Furthermore, gel electrophoresis and western blotting have identified bands with molecular masses at 130 KDa, 40KDa, 100KDa and over 170KDa respectively.PCR amplification of the conservative region of the VP60 gene of the virus confirmed the presence of a very clear band migrating about 300 bp. The results of nucleotide sequencing showed that the homology was 89-100% compared with the reference sequence published in Gen Bank.These results indicate that a convenient and robust system allow the study of RHDV after infection of cells *in vitro* and subsequently led to the identification of viral genomic organization by strong specific, high sensitive molecular tools which will undoubtedly aid in the study of the molecular mechanisms of RHDV replication, pathogenesis and epidemiology.

Key words: Rabbit Hemorrhagic Disease Virus · RT-PCR · Western Immunoblot · Electrophoresis · Sequence Analysis

INTRODUCTION

Rabbit hemorrhagic disease virus represents the causative agent of a lethal disease in rabbits that was first described in China [1], spread rapidly and emerged a few years later in other areas of Asia, Mexico, different European countries and elsewhere [2-4]. Severe necrotizing hepatitis and hemorrhages are the most commonly observed features. After a short incubation period of 36 to 48 h, the mortality rate can reach up to 100% in RHDV-infected rabbits older than 8 weeks of age [5-7]. The full-length genome of RHDV is a positive-stranded RNA virus which has been included as a member of the *Caliciviridae* family, a group of non-enveloped and icosahedral animal viruses designated by the International Committee on Taxonomy of viruses as the type species of the new genus *Lagovirus* [8-11]. Based on genetic and antigenic analyses, the RHDV species were divided into at least two subtypes, although antigenic variants have been reported, only one serotype has been identified to date [12-15]. RHD viral gene is identified as a virus with a genome of about 7.5 kb and a sub-genomic RNA of 2.2 kb. Sequence analysis of the RHDV genome revealed the presence of two open reading frames (ORFs) and a poly(A) tail. ORF1 encodes a polyprotein of 257 kDa that is predicted to be cleaved into several mature proteins,

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including an NTPase, a proteinase, an RNA-dependent RNA polymerase and the virion coat protein VP60 [8, 16, 17]. ORF2 is much smaller and encodes a single polypeptide VP2 of 12.7 kDa, a basic protein present in small amounts in virions [8,9,18-22]. The capsid protein VP60 is the main target of the host immune defence against RHDV and plays an important role in virus diagnosis and vaccine design. The viral capsid seems to result from the multimerization of 180 copies of a single VP60 protein and is also generated via cleavage of the ORF1-encoded polyprotein [17,23-26], which therefore appears to be a good candidate for the elicitation of a protective response [3]. In some cases the recombinant VP60 protein self assembled to form virus-like particles which were highly immunogenic [8,9,19,28,29]. There are two main hypothesises about the function of VP2 at present. One is that the VP2 protein might play a role in virus-particle assembly by interacting with both VP60 and the viral RNA (or the RHDV VPg protein linked to virus RNA), thus mediating specific encapsidation of the virus genome. The other idea is that it may be a regulatory factor related to virus replication, protein expression or virus-induced cell apoptosis, etc [30]. As RHDV can not be propagated well under tissue or cell culture conditions, much research progress in caliciviruses has been made by *in vitro* translation or by using the baculovirus system. So far, viral protein expression has only been studied by *in vitro* translation of viral RNA and detection of RHDV-encoded proteins with specific antibodies [16,17,27]. Protein analyses led to the detection of 13 RHDV-specific polypeptides within the infected rabbit hepatocytes, these proteins were assigned to defined regions of the viral genome, resulting in a refined model of RHDV genome organization [31]. However, a recombinant capsid protein VP60 expressed in the baculovirus/ Spodoptera frugiperda 9 insect cells (Sf9), cell expression system self-assembled into virus-like particles,these particles turned out to be highly immunogenic and to induce good protection against the disease [32,33]. Recently, an infectious cDNA clone of RHDV was constructed and the rescued virus is adapted to growth in rabbit RK13 cells, providing a useful platform for the study of RHDV [34]. The effect of VP2 on the expression levels of VP60 in mammalian cells was then tested by using RK13 cells transfected with recombinant plasmids containing the luciferase gene, this expression was confirmed by using an indirect immunofluorescence assay and western blot analysis [27]. VP2 was shown to increase the expression of capsid protein VP1 and to stabilize it and the presence of RHDV VP2 can increase virus replication [25,26]. Report experiments investigating the effect of VP2 on the expression of VP60 *in vitro* and *in vivo*, showing that VP2 is capable of down regulating the expression of VP60 [27]. The structural polypeptides of RHDV were investigated by SDS-PAGE, using 12% slab gels in a discontinuous buffer system, a major and several minor distinct polypeptide bands were visualized; molecular masses were approximately 60 kilodaltons (kDa) for the major component and 85, 56, 40 and 30 kDa for the minor components [3, 19, 35, 36]. Western blot analysis of such polypeptides with convalescent sera has been resulted in similar molecular weights [19, 27,37,38]. Many studies on genetic information on RHDV have been conducted [14,39-43]. The use of mabs in ELISAs or western immunoblots also enables distinction to be made between the related viruses of RHD and has contributed to antigenic characterization studies [37,38,44]. The novel approaches of molecular epizootiology, which involve the application of PCR and rapid characterization of the amplicons by restriction enzyme analysis or sequencing, have greatly improved the detection and characterization of viruses. RT-PCR which can provide significant clinical diagnosis as a modern molecular biological tool, can be used in detection of large numbers of samples and investigation of RHD epidemiology [45-50]. The results showed that specificity and the sensitivity of the RT-PCR was 1 x 10^4 times higher than that of the hemagglutination assay and was able to detect RHDV in all viscera, but not in feces. A reverse genetic system has been crucial for the production and characterization of RHD viral proteins because their high rate of genetic variation can play an important role in the emergence of a disease. Here, we present information on a genetic analysis of vitro expression of RHDV proteins in infected RK 13 cell culture to determine the extent of change in field isolates and whether they have significant differences from the previously characterized worldwide RHDV isolates.

MATERIALS AND METHODS

Cell Culture and Virus Propagation: Liver specimens collected from rabbits infected with RHD in various farms were used and diagnosis was initially confirmed by the HA test. Livers from infected rabbits were homogenized and virus particles purified from the clarified suspension as previously described [45]. RK-13 cells obtained from tissue culture unit in the holding company of biological products and vaccines (VACERA) were grown in monolayers with minimal essential medium containing 5% fetal calf serum; 100 IU/ml penicillin; 100 µg/ml streptomycin; 100 µg/ml gentamicin; and 5 µg/ml

amphotericin (fungizone) in a humidified atmosphere of 5% CO2 at 37°C. Cells (5×10^5 cells/well) were seeded in tissue culture flasks (Nunc and Life Technologies) and grown to 80% confluence. For virus propagation, tissue culture flasks showing confluent cell growth were inoculated with the clarified suspensions containing virus particles, incubated for 24-72 h at 37°C and daily examined for a pronounced cytopathic effect [51]. Supernatants from both non-inoculated and inoculated RK-13 cell cultures were basically used for subsequent genetic analysis. genetic C

Dual Extraction of the Genomic RNA and Viral Proteins: Both genomic RNA and viral proteins were extracted from a 0.5 mL sample of each strain [52], using Trizol reagent (Life technology cat # 15596) according to the instruction of the manufacture.

First, 0.5 ml of Trizol reagent was added to 0.5 ml of each strain and incubated at 25°C/30min and then 200µl of chloroform was added and incubated for 3 min before centrifugation at 14000rpm /15min at 4°C. The upper aqueous phase was completely collected and precipitated by adding 0.5ml of absolute ethanol and centrifuged as before.

Extraction and Purification of Genomic RNA: The precipitated RNA washed twice with 0.1M sod. citrate in absolute ethanol and finally redissolved in 50ul of 8 mM NaOH. The pH was then adjusted at 8 by adding 115 µl/ml HEPES (0.1 M).

Extraction and Purification of Viral Proteins: The protein rich supernatant was transferred to another 1.5 microfuge tube and the protein was precipitated with double volume isopropyl alcohol and sedimented by centrifugation as before. Protein was then washed twice with 0.3 M guanidine hydrochloride in 95% ethanol, centrifuged as before. The protein pellet was then redissolved in 100µl of PBS and stored at-20°C till used (52*)*.

Determination of Protein Concentration: The protein concentration was estimated using modified lowery method [53].

Sodium Dodecyl Sulphate-Polyacrylamide GEL Electrophoresis (SDS-PAGE): Extracted proteins of each strain were resolved on discontinuous buffer system composed of 10% (w/v) acrylamide separating gel and 4% stacking gel [35]. Electrophoresis was carried out at a constant voltage (100 V) until the bromophenol blue dye moved to the bottom of the gel. The gels were stained with coomassie brilliant blue for 2 hours and destained overnight. Molecular weight of each protein band was calculated with reference to a standard curve derived from the migration pattern of standard prestained molecular weight markers (Page Ruler, Fermentas cat#SM0671).

Western Immunoblotting Analysis: The electrophoretic transfer of polyacrylamide gel resolved proteins to the nitrocellulose membrane was carried out by electroblotting as described [54] using BioRad Electro Transfere unit. The unoccupied sites on the nitrocellulose membrane were blocked with blocking buffer (Tris buffered saline (TBS), pH 7.2 containing 0.1% Tween-20, 1% (w/v) western blot grade gelatin and 0.05% Triton X100). The nitrocellulose membrane was then incubated with rabbit Anti *RHDV* antisera (1:500 in blocking buffer) at 37°C for 1 hour followed by washing three times with TBS-Tween 20. The membrane was then incubated at 37°C for 1 hour in anti-rabbit peroxidase labeled dilution of 1:5000 in TBS-tween 20. The membrane was then washed as above and incubated in freshly prepared substrate solution (10 mg aminoethyle carbazone in 2.5 ml Isoamyleformamide and 47.5 mL of acetate buffer pH 5to which 50 µl of 30 % H_2O_2 was added) for 3-4 min for color development and visible bands were developed, then the reaction was stopped by washing the membrane with running distilled water. Page Ruler prestained protein ladder (Fermentas cat#SM0671) was used in this experiment.

PCR Amplification: A primer was designed to amplify the conservative region of VP60 gene using DNASTAR® V9 software. The PCR was performed in 50-µl reaction mixtures containing 25µL of green Dream Taq master mix (fermentase Cat # K 1081) and 50 pmol of the forward primer (5'-CGA-CGT-TGT-AAA-ACG-ACG-GCC-AGT-GTT-ACG-ACT-GTG-CAG-GCC-TAT-GAG-TT-3') and the reverse primer (5'-TTG-TTG-AGC-GAA-AGT-CCA-ATT-GTC-ACT-G-). The M13 forward sequencing sequence was added 5' upstream the sense primer to facilitate the sequencing reaction. Thermal cycling was performed using T professional, thermal cycler (Biometra, Germany), the parameters for amplification were denaturation at 95 °C for 3 min and 40 cycles at 95°C for 1 min, 60 °C for 45 sec and 72°C for 1 min. A final extension at 72 °C for 10 min was also included. The amplicon was electrophoresed on 1% agarose. 100pb plus DNA ladder (100 pb ladder, SibEnzyme) was used to calculate the exact amplicon size.

Sequencing: The complete nucleotide sequences of the VP60 conservative gene of two serovars were performed in Macrogen USA. For preparation of the gene for sequencing, the PCR product was separated on 1% low melting agarose and electrophoresed on low voltage (20 V) at 4°C. The bands were sliced off and purified with the Biospin PCR purification kit (Biobasic cat # BSC03S1) as described by the manufacture. Briefly, the gel slices were melted at 60°C for 5 min, mixed with 500 ml of gel extraction buffer and placed on the biospin column provided with the kit, centrifuged at 4000 rpm/2min/4°C and washed twice with the washing solution. Finally the amplicon was eluted in 50 µL of the elution buffer and stored at-20 °C till sequenced. Sequencing reactions were performed in a MJ Research PTC-225 Peltier Thermal Cycler using ABI PRISM 3730XL Analyzer BigDyeTM Terminator Cycle Sequencing Kits with AmpliTaq DNA polymerase (FS enzyme Applied Biosystems), following the protocols supplied by the manufacturer. Single-pass sequencing was performed on each template using the primer used for PCR amplification. The fluorescent-labeled fragments were purified from the unincorporated terminators with an ethanol precipitation protocol. The samples were resuspended in distilled water and subjected to electrophoresis in an ABI 3730xl sequencer (Applied Biosystems).

RESULTS AND DISCUSSION

In the present study, examination of RK-13 cell cultures 24-72 h after infection with RHDV for cytopathic lesions revealed signs of degeneration, including condensed nuclei considerably smaller than control ones. The infected cells were significantly increased in cultures maintained for 72 h after infection. The findings in this investigation stand in marked agree to Alexandrow. *et al.* [55] who demonstrated that these cells can be infected *in vitro* which may suggest the replication of the viral genome and/or transcription of the subgenomic mRNA could take place in infected cell cultures. In RHDV, the translation of viral RNA in rabbit reticulocyte lysates resulted only in a protease-polymerase protein of 69 kDa [17]. However, the 15 kDa protease and 58 kDa polymerase could additionally be detected in cultures of RHDV infected primary rabbit liver cells [31]and also after transient expression of RHDV cDNA in cell culture [21]. Additionally, Liu *et al.* [34] pointed out that the absence of a robust cell culture model of RHDV infection has severely limited analysis of the RHDV life cycle and the development of effective antivirals and vaccines. Herein,we analyzed the expression of RHDV proteins in infected RK-13 cultures by SDS-PAGE. The structural polypeptides of RHDV were investigated by SDS-PAGE, using 10% (w/v) acrylamide separating gel and 4% stacking gel in a discontinuous buffer system and their approximate molecular sizes were estimated by direct comparison with reference markers after Coomassie blue staining. Major bands migrating about 130 KDa and 40KDa and several minor distinct protein bands at 100KDa and over 170KDa were visualized (Fig. 1).These data showed that the structural protein of RHDV was mainly represented by a single major polypeptide species of 130 kDa, which constitutes a distinctive property of caliciviruses that is not shared by other small viruses of vertebrate origin.

Concerning structural proteins, the major capsid protein VP60 and the minor basic structural protein VP2 have both been described as components of calicivirus particles which are all found in virions [8,17,56-61].In experiments expressing the RHDV capsid protein, this basic protein plays a role in packaging of the viral RNA or in infectivity. The composition of this protein of mainly basic amino acids would support the speculation that it functions as nucleic acid-binding protein [17,18].For the investigation of viral proteins, Supernatants from inoculated RK-13 cell cultures were analysed on western blots.The viral polypeptides were separated by SDS-PAGE, electrotransferred onto a nitrocellulose membrane and probed with hyperimmune sera prepared against

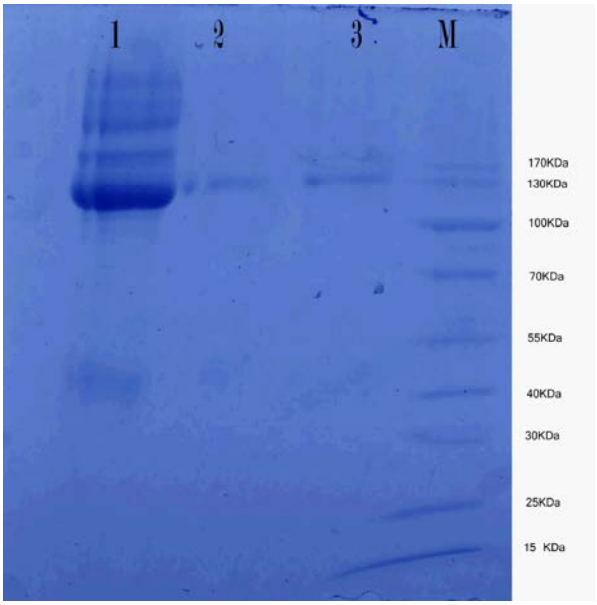

Fig. 1: SDS-PAGE analysis of RHDV propagated in RK-13 cell culture (lane 1). Lanes 2 and 3 are the negative controls. M is the molecular weight marker.

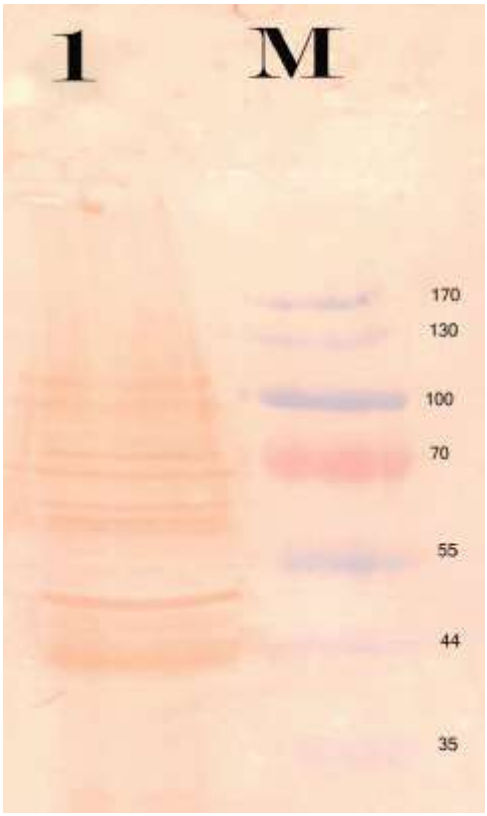

Fig. 2: Western blotting analysis of RHDV propagated in RK-13 cell culture and sampled after infection with clarified viral suspension (lane 1). M is the molecular wight marker.

the RHDV (Fig.2).Antibodies were raised against parts of the viral proteins and western analysis has identified a polypeptide with band of 130 KDa, which may represent a degradation product of the VP60 main antigen or it may be a minor component of the virion. These results are in consistent with those from Schaffer and Soergel, [23] who confirm that a single apparently virus-specific protein could be detected in fractions examined with SDS-PAGE and western blot shown to contain virus particles that cause RHD and resemble caliciviruses. It is well known that the use of reverse genetic systems is a useful tool for studying RNA virus replication, pathogenesis and *in vivo* function of individual viral proteins as well as for developing new vaccines, therefore, a novel diagnostic RT-PCR systems with high levels of sensitivity and specificity is highly needed. The VP60 gene was chosen as the target for the diagnostic PCR assays, because parts of this gene from RHD samples have been sequenced [39,62]. In the current research, RT-PCR was performed using specific primers specific for the RNA polymerase, the banded amplicons of the conservative region of

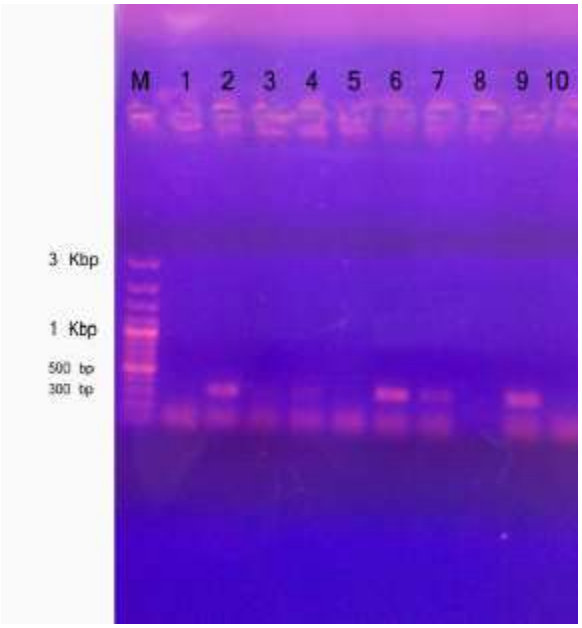

Fig. 3: PCR amplification of the conservative region of the VP60 gene of the RHDV propagated on RK-13 cell culture. lane 1 (non-infected tissue culture) and lane 2-7 (infected tissue culture). lane 9 and 10 are respectively positive and negative controls. M is the 100pb plus marker

the VP60 gene of the RHDV applied on supernatants from RK-13 cell cultures were presented in Figure 3., whereas a very clear band migrating about 300 bp was detected. RT-PCR based detection of viral RHDV RNA has been shown to be rapid, sensitive, special, efficient and has been successfully used to analyze the RHDV genome [12,48,63] Consequently, our study noted that RT-PCR enable the screening of cell culture to detect the presence of viral RNA in a biological material indicating the sensitivity of the PCR.The two sequences of the banded amplicons of supernatants from inoculated RK-13 cell cultures are shown in figure 4 and 5. The nucleotide sequence of the amplified region of the VP60 gene from the liver samples has been described as Rabbit hemorrhagic disease virus isolate GIZA-2006 VP60 (VP60) gene, partial cds >emb|HE963222.1| Rabbit haemorrhagic disease virus mRNA for VP60 protein (VP60 gene) under accession number JQ995154.1., table 1. Regarding genomic organization, the investigation also revealed considerable homology (89-100%) to the sequences of other isolates originating from various countries.The relationship to the calicivirus family was also evident in the conservation of several amino acid motifs distributed throughout the genome, with the same linear arrangement and relative distances. In parallel to our findings,

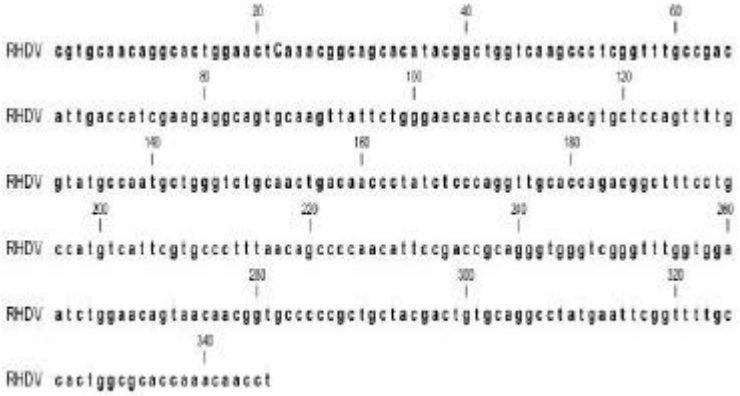

Fig. 4: The nucleotide sequence of the conservative region of the VP60 gene of RHD

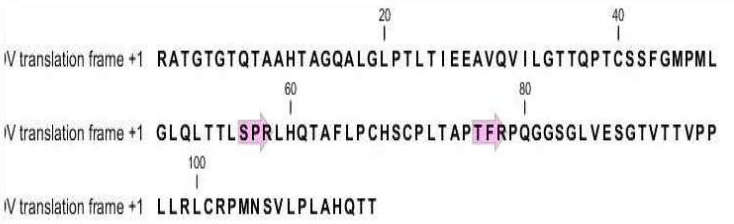

Fig. 5: The deduced amino acid sequence of the conservative region of the VP60 gene of RHDV

Table 1: Blast analysis of the conservative region of the VP60 gene of RHDV

Accession	Description	Query coverage	E value	Max ident
JX409902.1	Rabbit hemorrhagic disease virus isolate KS2000 structural protein VP60 gene, partial cds	100%	6e-172	99%
JQ995154.1	Rabbit hemorrhagic disease virus isolate GIZA-2006 VP60 (VP60) gene, partial cds >emb| HE963222.1| Rabbit haemorrhagic disease virus mRNA for VP60 protein (VP60 gene)	100%	6e-172	99%
GU564448.1	Rabbit hemorrhagic disease virus capsid protein VP60 gene, complete cds	100%	1e-158	96%
EF558583.1	Rabbit hemorrhagic disease virus strain Triptis, complete genome	100%	1e-158	96%
Y15442.1	Rabbit haemorrhagic disease virus VP60 gene, isolate Triptis	100%	6e-157	96%
EF558584.1	Rabbit hemorrhagic disease virus strain Rossi, complete genome	100%	3e-155	96%
AF453761.1	Rabbit hemorrhagic disease virus capsid structural protein VP60 (vp60) gene, complete cds	100%	3e-155	96%
EU250330.2	Rabbit hemorrhagic disease virus isolate Pv97 capsid structural protein VP60 (VP60) gene, complete cds	100%	1e-153	95%
EU003582.1	Rabbit hemorrhagic disease virus isolate UT-01, complete genome	100%	1e-153	95%
AJ302016.3	Rabbit hemorrhagic disease virus VP60 gene for capsid protein, genomic RNA, isolate 99-05	100%	1e-153	95%
DQ205345.1	Rabbit hemorrhagic disease virus strain JX/CHA/97 polyprotein (RHDV), major capsid protein VP60 (VP60) and structural protein VP12 (VP12) mRNAs, complete cds	100%	6e-152	95%
JN851733.1	Rabbit hemorrhagic disease virus isolate Klyazmenskiy-2004 polyprotein (VP60) gene, complete cds	100%	3e-150	95%
EU250331.1	Rabbit hemorrhagic disease virus isolate Vt97 capsid structural protein VP60 (VP60) gene, partial cds	100%	3e-150	95%
AF258618.2	Rabbit hemorrhagic disease virus isolate Iowa2000, complete genome	100%	3e-150	95%
AJ969628.1	Rabbit hemorrhagic disease virus VP60 gene for capsid protein, genomic RNA, isolate 03-24	100%	1e-148	95%
AJ303106.2	Rabbit hemorrhagic disease virus VP60 gene for capsid protein, genomic RNA, isolate 00-Reu	100%	1e-148	95%
DQ841708.1	Rabbit hemorrhagic disease virus isolate CUB5-04 polyprotein gene, partial cds	99%	5e-148	94%
FJ794180.1	Rabbit hemorrhagic disease virus isolate WF/China/2007 capsid protein (pol) gene, partial cds	100%	6e-147	94%
EF558582.1	Rabbit hemorrhagic disease virus strain Dachswald, complete genome	100%	6e-147	94%

Oehmig *et al*. [64] described the overall genomic organization as well as the nucleotide sequence of a calcivirus isolate which is in contrast to previous suggestions from other researchers who have analysed sequence variability within the whole RHDV genome or its fragments [14, 15, 42,43, 65-67].To summarized the findings in this investigation, results showed that *in vitro* propagation systems could provide precious information in the study of genetic expression and replication of caliciviruses in cell cultures. Besides, The raise in the average homology similarity 89-100% upon the passage of the RHDV recovered from the field could raise the adaptability of the virus to the host upon passaging which could result in higher pathogenicity. In any case, the infection of RK-13 cell cultures represents a means of analyzing RHDV gene expression on the basis of infected cells. RT-PCR assay described in this paper, in combination with rapid characterization of the PCR products by sequencing, have been evaluated as a practical approach for the rapid and sensitive methods for identifying RHDV samples and estimating the phylogenetic relationship to previously sequenced RHDV specimens which has clarified the considerable stability of the virus characteristics without any amino acid mutation. Finally, regular analysis of various strains may be of practical importance in the control of an outbreak by the rapid molecular identification of the responsible agent as a future comparative work to observe the genetic evolution of the virus as it adapts to the surrounding environment.

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