

## IDENTIFICATION AND CHARACTERIZATION OF THE CURRENTLY CIRCULATING RABBIT HEMORRHAGIC DISEASE VIRUS ISOLATES

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### ABSTRACT

Even among vaccinated rabbits, the virus that causes rabbit viral hemorrhagic disease (RHDV) has been increasing in Egypt recently. So, the current study screened the emergent RHDV of vaccinated and unvaccinated domestic rabbits in some Egyptian provinces between 2022 and 2023. 40 pooled Samples were collected from different rabbit flocks with age range of 2 months up to 4 months with high mortality rates, clinical symptoms, and post-mortem lesions related to RHDV. Hemagglutination (HA) test, reverse transcriptase-polymerase chain reaction (RT-PCR) targeting the partial VP60 and sequencing and phylogenetic analysis were conducted for genotyping of RHDV strains. The results revealed that 16 out of 40 cases that were positive for RHDV and were hemagglutinin-positive, with titer ranging from 2<sup>3</sup> to 2<sup>14</sup>. Randomly six isolates were selected for genetic sequence and phylogenetic analysis which revealed their clustering with RHDV<sub>2</sub> strains which were detected for the first time in Upper Egypt and submitted on GenBank as (OQ925947 Assiut .vac1 to OQ925952 Assiut .vac6). The nucleotide sequence identities of the six isolates were 98.8-100 % compared to each other. The recently isolated strains had nucleotide difference 23, 1 % when compared to commonly vaccinal strain (JQ995154 Giza 2006). The inoculated rabbits expressed RHDV-typical signs and postmortem findings and Ninety percent (90 %) mortality rate was recorded within 3–6 days post-infection. Microscopic examination revealed that presence of acute necrotizing hepatitis, congestion and widespread hemorrhages in all internal organs; liver, lungs, spleen, kidneys and brain. In conclusion, the presence of RHDV-2 strains was detected for the first time in some Upper Egypt provinces.

**Key words:** RHDV<sub>2</sub>, RT-PCR, VP60, Sequencing, Histopathology.

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### INTRODUCTION

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Rabbit viral hemorrhagic disease (RVHD) often referred to as rabbit plague and rabbit hemorrhagic pneumonia is caused by the rabbit hemorrhagic disease virus (RHDV), a Lagovirus belonging to the Caliciviridae family (Vinjé *et al.*, 2019). It has a detrimental impact on Egypt's economy, resulting in losses in the country's rabbit

output due to its high morbidity and mortality (Mohamed, 2009; Fahmy *et al.*, 2010). RHDV is a naked, icosahedral, single-stranded, positive-sense RNA virus (OIE, 2021; Ismail *et al.*, 2017). its nucleic acid includes two open reading frames (ORFs); ORF1 and ORF2. The ORF1 encodes non-structural proteins; the RNA-dependent RNA polymerase and the major capsid protein (VP60). ORF2 encodes the minor structural protein vp10 (Dalton *et al.*, 2015; Meyers *et al.*, 2000). VP60 capsid protein is the main structural protein of RHDV, which contains the type-specific antigenic epitope (Capucci *et al.*, 1998). There are three distinct RHDV groups: “classical” RHDV with the genogroups G1–G5, the antigenic variant RHDVa/G6 (Le Gall Recule *et al.*, 2003), and the new type RHDV2/RHDVb (Le Gall Recule *et al.*, 2013). The average nucleotide identity between RHDV2 and RHDV1 (RHDVa) can reach 82.4%, with amino acid similarity of about 89.2% (Kong *et al.*, 2016). Different RHDV variants belonging to GI.1 were identified in vaccinated (Metwally and Madbouly, 2005 and El Sissi and Gafer, 2008) and non-vaccinated rabbits (Magouz *et al.*, 2019) despite several vaccination programs. Recently, RHDV strains relating to GI.2 (RHDV2/b) have been identified in vaccinated rabbit flocks (Abodalal and Tphoon, 2020).

Initially, RHDV was discovered in China in 1984, then quickly spread over the entire world (Liu *et al.*, 1984) and became endemic in several regions (Abrantes *et al.*, 2012). In 2010, the RHDV2 variant was recognized in France in rabbits of different ages and populations (Le Gall-Recule *et al.*, 2011). The first case of RHDV in Egypt was recorded in 1992 in Sharkia Province (Ghanem *et al.*, 1992). The subsequent incidence of the RHD occurred in Assiut Province during the winter of 1992 (Salem and Ibrahim, 1992).

RHD causes high abrupt deaths preceded by bloody frothy nasal discharge in the different rabbit breeds and/or neurological signs (Trzeciak-Ryczek *et al.*, 2015) RHDV is characterized by acute necrotizing hepatitis and the production of disseminated intravascular coagulation that produces wide

spread hemorrhages in all internal organs; liver, lungs, heart, kidneys and the trachea (Abrantes *et al.*, 2012; Embury-Hyatt *et al.*, 2012).

Inoculating susceptible rabbits, genomic identification, and characterization of the virus are required for virus detection since the virus cannot be cultivated in cell cultures (OIE 2021; Ismail *et al.*, 2017). The molecular detection of the genetic variations between RHDV strains was carried out on partial and complete sequences of the VP60 gene (El Bagoury *et al.*, 2014; Wang *et al.*, 2012).

In comparison to serological techniques, RT-PCR is thought to be a fast and accurate diagnostic procedure for RHDV (Soliman *et al.*, 2016). Pathological investigation plays an important role in identification of viral infection in Rabbit diseases (Hamed *et al.*, 2013).

Continuous monitoring of RHDV circulating in Egypt is an urgent demand for effective control of RHDV outbreaks. So, this study's objectives included isolation, molecular identification, and characterization of the currently circulating RHDV in Egypt during 2022-2023 by sequencing the partial VP60 protein and comparing the circulating isolates with the vaccinal strains.

## MATERIALS AND METHODS

### Ethics statement

The Institutional Animals Care and Use Committee, Research Ethics Board, Faculty of Veterinary Medicine, Assiut University (No. 06/2023/0060) approved the study protocols, following animal welfare guidelines.

### Study area

The study area represents some northern regions of Egypt (Alexandria, ELBeheira, Aldakahli, ELGharbia, Kafr El-Sheikh, ELSharqia, Damietta, and Cairo) and Southern Egypt includes Assiut, and New Valley (Fig. 1).



Fig. 1: Egypt map displaying the geographical distribution of the study

### Sampling

Between June 2022 and March 2023, forty pooled liver specimens of RHD-suspected rabbits (*Oryctolagus cuniculus*) were collected from backyard rearing and farms. The examined rabbits were housed in wire cages and fed on the commercially prepared pelleted diet. The animals under investigation varied in age and vaccination status as demonstrated in Table 1. From the history of investigated farms, the owners from Upper Egypt confirmed that they imported new

rabbit breeds from the Lower Egypt to upgrade their farms with new breeds.

All specimens were gathered aseptically from the freshly dead animals in separate sterile plastic bags and transported on dry ice to the laboratory of Avian and Rabbit Medicine Department, Faculty of Veterinary Medicine, Assiut University. Specimens were preserved at  $-20^{\circ}\text{C}$  till virologic and laboratory analysis (OIE, 2021).

Table 1: History of investigated farms.

Governorate	Date	Vaccination status	age	No.of samples
ELBehira	16/6/2022	Un-vac.	breeder	3
ELGharbia	15/8/2022	*Vac.	growing	2
New valley	8/9/2022	Un-vac.	growing	6
Assiut	20/9/2022	Un-vac.	growing	3
ELSharkia	2/10/2022	*Vac.	breeder	4
Alexendria	5/12/2022	Un-vac.	growing	3
Assiut	12/1/2023	*Vac.	breeder	3
Damietta	20/2/2023	Un-vac.	growing	3
Assiut	20/3/2023	*Vac.	breeder	3
Cairo	31/3/2022	*Vac.	breeder	4
El dakahlya	12/11/2022	Un-vac.	growing	6

Growing rabbits aged 55 days up to 4 months, and adult rabbits aged more than 4 months.

\*Imported RHDVa vaccine

**A: Specimens processing:**

Aseptically, the liver tissue was mechanically homogenized by using a pestle tissue grinder and suspended in 10% (w/v) phosphate-buffered saline (pH 7.2). The prepared suspension was alternately frozen and thawed three times for viral release, centrifuged at 5000 rpm for 15 min. The clear supernatants were harvested and stored at -80 °C till used (OIE, 2021; Daodu *et al.*, 2021).

**B: RHDV Screening:****I. Haemagglutination screening:**

RHDV in the clarified liver homogenate was screened by investigating its hemagglutinating properties using “rapid slide” and “microplate” hemagglutination (HA) assays adopting O-type human erythrocyte (OIE, 2021).

**i. Erythrocyte preparation**

Four parts of freshly drawn O-type human blood samples were added to one part of 4% sodium citrate (as an anticoagulant). The erythrocytes were washed in an equivalent volume of PBS (1:1 v/v) and then centrifuged at 500 g for 10 min. The supernatant was discarded, and the washing step was repeated twice (OIE, 2021; Magouz *et al.*, 2019).

**ii. Rapid slide HA**

The supernatants of all liver specimens were rapidly checked for HA activity following Du, (1990) methodology. On a sterile clean and dry glass slide, one drop of the liver homogenate was gently well-mixed with one drop of 10 % (1:9 v/v) PBS-suspended erythrocytes within a minute, the agglutination degree was recorded.

**iii. Micro plate HA**

The viral HA titer in all supernatants (40 specimens) was measured by the quantitative “microplate HA” assays documented by Capucci *et al.* (1996) and the OIE (2021). In round-bottom microtiter plates, double-fold serial dilutions of each liver extract were prepared in 50 µl PBS (7.2 pH). Then, an equal volume of 0.75% washed erythrocytes

was added to each dilution and incubated at 4 °C.

After 20-30 minutes, the wells with lattice and button shapes were recorded for each specimen (Salman *et al.*, 2010 and OIE, 2021).

The HA titer was recorded at the highest virus dilution that agglutinated the RBCs (i.e. the last well displayed full HA). Haemagglutinating units were expressed as Log<sub>2</sub> and (4 HA unit) was considered negative as recommended by OIE, (2021).

Positive and negative controls along each plate were considered. Each sample was tested in three replicates. The specimens were considered positive when the dilution of the agglutination endpoint was > 1/16 (2<sup>4</sup> HA unit/50 µl) as described in the OIE, (2021).

**II. Molecular RHDV identification****i. One step Reverse transcription PCR (RT-PCR)**

RT-PCR was performed by using RNA extracted from the clarified tissue homogenate of all specimens using the viral RNA Mini kit (Qiagen, Gmbh, Germany catalogue No. 52904). The VP60 c-terminal region (538 bp) was partially amplified using the RHDV-specific primer set [P33: CCACCACCAACACTTCAGGT and P34: CAGGTTGAACACGAGTGTGC (Metabion, Germany)], Rt-PCR Master mix (Qiagen, Gmbh, Germany), and one step reverse transcriptase in a Bio-Metra thermal cycler following Fahmy's *et al.*, (2010) cycling condition. Accurately, RT-PCR was done at 50°C for 30 min, then a primary hot start at 95°C for 10 min, followed by 35 cycles each consisting of a denaturation step at 94°C for 30 s, annealing at 56°C for 40 s, and extension at 72°C for 45 s. A final over-extension step at 72°C was performed for 10 min. The amplicons were electrophoresed using 1.5% agarose gel (Invitrogen, Thermo-Fisher Scientific, Germany) stained with 0.5 µg/ml ethidium bromide at 100 V for 45 min and visualized under a UV transilluminator. The amplicon size was measured by a 100-

1000 bp DNA ladder (Cat.no.SM0243, Fermentas). Two controls were included in each amplification run: a positive control and a negative control (NAase-free water).

## ii. Gene sequencing

The RT-PCR products of 6 randomly selected were purified from the gel by using a PCR Product Recovery kit (Qiagen, Gmbh, Germany). The purified PCR products were directly sequenced by using a BigDye Terminator v3.1 cycle sequencing kit (Perkin-Elmer, Fostercity, CA) and the same primer set. The sequences were produced using a 3130 genetic analyzer (Applied Biosystems, Life Technologies, Thermo-Fisher, Germany). Samples were carefully selected from various locations of governorates representing Lower Egypt (Beheira and Gharbia) and Upper Egypt (Assiut and New Valley)

The produced sequences were identified via nucleotide Blasting against the sequences available on GenBank of the National Center for Biotechnology Information, NCBI. The assembled VP60 sequences were uploaded to GenBank for obtaining accession numbers.

## iii. Sequence alignment and phylogenetic analysis

For achieving the molecular analysis, the datasets of VP60 nucleotide sequences were aligned against the closely related sequences published on GenBank (Coordinators, 2016) using the Clustal W software with the default options.

A phylogenetic tree was constructed for the obtained sequences using the Neighbor-Joining distance method with the p-distance model and maximum-likelihood (ML) scheme (Saitou and Nei, 1987) with the Tamura *et al.* (2013) model, both with at least 1000 bootstrap replications in MEGA (version 6) software.

## III. Assessing RHDV pathogenicity

### i. The virus

The Six-sequenced RHDV isolates were used as infecting viruses. All isolates were

propagated separately through the passage in RHDV-seronegative susceptible-rabbits (3 rabbits/isolate) as reported previously elsewhere (Abd El-Moaty *et al.*, 2014). Under aseptic conditions, livers were collected from the infected rabbits and 10 % clarified liver homogenates (in 7.2 pH PBS) were prepared from each isolate. The homogenates were filtered through 0.45 µm filters and decontaminated with adding penicillin-G-sodium, streptomycin, and clotrimazole (100 IU/ml, 100µg/ml, and 100 µg/ml) and incubating for 1 hour at the room temperature.

### ii. Rabbits and experimental design

Thirty-five 2.5 kg 3-month-old New-Zealand rabbits were obtained from the laboratory animal center, Faculty of Veterinary Medicine, Assiut University. All rabbits were free from detectable RHDV antibodies as examined by hemagglutination inhibition (HI) test according to El-Nahas, (2011). The animals were divided into 2 groups. The first group was subdivided into 6 sub-groups, 5 rabbits/each. Each sub-group was intramuscularly inoculated with 1-2 ml of the 10% liver homogenate related to the mentioned isolates. The 2<sup>nd</sup> group (5 rabbits) was kept as a negative control. The animals were observed for any clinical and postmortem changes and mortality till a week post-infection (Ferreira *et al.*, 2004). At necropsy 1 cm liver, lung, spleen, brain and kidney specimens were formalin-fixed (10 % Neutral buffer), processed, and stained with hematoxylin and eosin according to the standard procedures (Bancroft *et al.*, 2013) for histopathological examination.

## RESULTS

### I. Signs of the RHD-suspected rabbit and postmortem lesions:

Rabbits under examination had signs and alterations that were consistent with RHD. They expressed nervous signs and frothy bloody nasal discharge followed by mortality. Various mortality rates were experienced.

Necropsy showed wide lesion distribution in most organs. A friable pale liver with hemorrhages accompanied by enlarged hemorrhagic and congested internal organs

(heart, thymus, lungs, spleen, and kidneys) was observed (**Fig. 2**). Hyperemic trachea with frothy exudate and cecal serosal petechiae were observed.

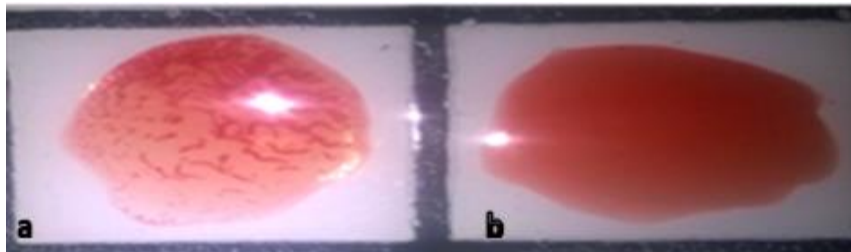


**Fig. 2:** Rabbit suspected to be infected with RHDV showing; **a.** nervous signs (convulsions), **b.** bloody nasal discharge, **c.** kidney and spleen were enlarged and congested, intestinal congestion and urinary bladder distended with colored urine, **d.** liver was enlarged size and pale with reticular pattern of necrosis and hemorrhages

## II. Detection rate of RHDV:

### i. HA test

Out of the 40 pooled specimens, 28 (70 %) showed positive HA reactions with titers ranging from  $2^3$  to  $2^{14}$  (**Fig.3**)

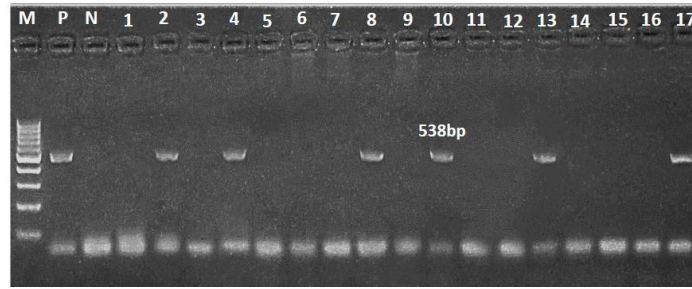


**Fig (3):** Rapid slide hemagglutination test. (A) showed positive HA while (B) showed negative HA activity.

### ii. Genetic detection of RHDV

The RT-PCRs detected the typical band of the RHDV-specific VP60 gene (538bp) (**Fig.4**) in 16 (40 %) specimens of all examined

samples. Twelve cases from vaccinated farms and four cases from unvaccinated farms were proved positive cases.



**Fig. 4:** Agarose (1.5%) gel electrophoresis showing amplification of a 538-bp fragment of the VP60 gene. **Lane M:** DNA ladder marker (1000 bp), **Lane P:** positive control, **Lane N:** negative control, **Lanes 2, 4, 8, 10, 13, and 17 are** positive specimens, and **Lanes 1, 3, 5, 6, 7, 9, 11, 12, 14, 15 and 17** are negative specimens.

**iii. Sequence alignment and Phylogenetic analysis:**

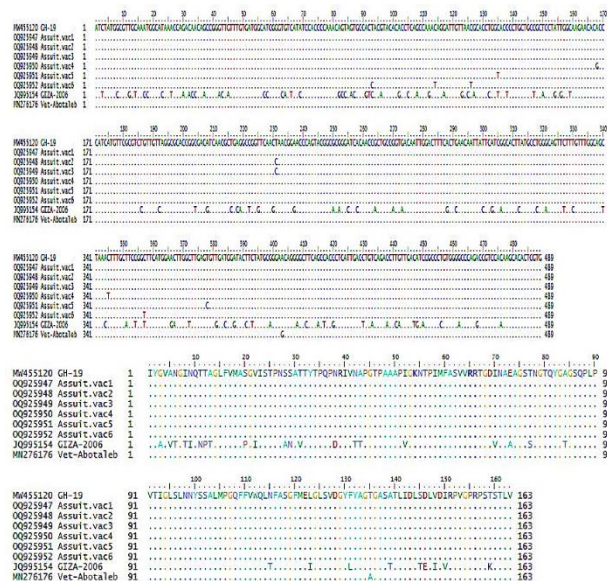
Nucleotide BLAST analysis in conjunction with VP60 sequencing data showed the close relation of these RHD-positive samples to RHDV2 (RHDV-GI.2). Six sequences were obtained and submitted to the GenBank under the accession numbers: OQ925947 Assiut. vac1, OQ925948 Assiut. vac2, OQ925949 Assiut .vac3, OQ925950 Assiut. vac4, OQ925951 Assiut. vac5, and OQ925952 Assiut .vac6.

The obtained alignment dataset is shown in **Fig.5.** The nucleotide sequence identities of the sequenced six isolates were 98.8-100 % compared to each other. A 93.7-99.8%

identities to MK629991tn-2018, MN276176 vet-Abotaleb, respectively, and 100% to other available RHDV2 strains (MW679028, MW455120GH-19, MW455122kfs-1-19, W455123kfs-219, MW455124BH-K-19, MW455125Alex-19) were observed.

The obtained isolates had 76.9% identity to JQ995154Giza 2006.

The alignment of 160 amino acids of RHDV-2 variant isolates compared to MW455120GH-19, MN276176vet-Abotaleb, and JQ995154 Giza 2006 available on the GenBank with their details listed in (**Fig. 5and 6**)was conducted.



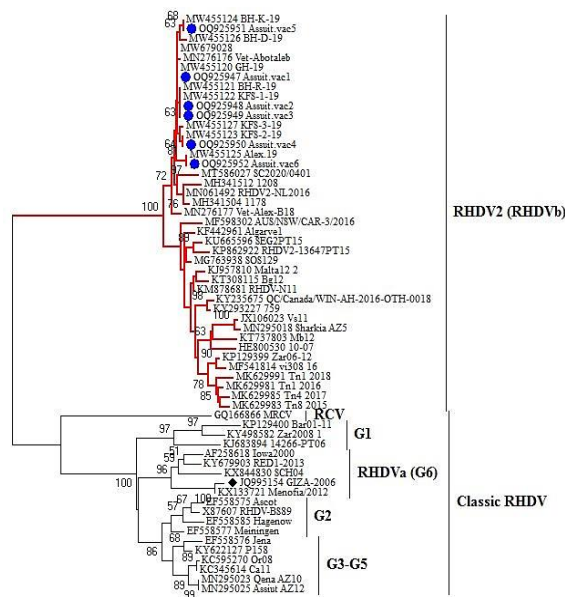
**Fig. 5:** Deduced amino acids and nucleotides alignment of 538bp fragment of VP60 gene of RHDV-2 isolates. The isolates belonging to RHDV-2 strains

		Percent identity																																			
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28								
Divergence	1	100	98.8	99.0	99.2	99.1	99.0	98.9	98.8	98.7	98.6	98.5	98.4	98.3	98.2	98.1	98.0	97.9	97.8	97.7	97.6	97.5	97.4	97.3	97.2	97.1	97.0	96.9	96.8	96.7	96.6						
	2	0.2	100	99.1	99.3	99.4	99.3	99.2	99.1	99.0	98.9	98.8	98.7	98.6	98.5	98.4	98.3	98.2	98.1	98.0	97.9	97.8	97.7	97.6	97.5	97.4	97.3	97.2	97.1	97.0	96.9	96.8					
	3	0.4	0.1	100	99.2	99.4	99.5	99.4	99.3	99.2	99.1	99.0	98.9	98.8	98.7	98.6	98.5	98.4	98.3	98.2	98.1	98.0	97.9	97.8	97.7	97.6	97.5	97.4	97.3	97.2	97.1	97.0	96.9				
	4	0.2	0.1	0.1	100	99.3	99.5	99.6	99.5	99.4	99.3	99.2	99.1	99.0	98.9	98.8	98.7	98.6	98.5	98.4	98.3	98.2	98.1	98.0	97.9	97.8	97.7	97.6	97.5	97.4	97.3	97.2	97.1	97.0			
	5	0.3	0.2	0.4	0.1	100	99.1	99.3	99.4	99.3	99.2	99.1	99.0	98.9	98.8	98.7	98.6	98.5	98.4	98.3	98.2	98.1	98.0	97.9	97.8	97.7	97.6	97.5	97.4	97.3	97.2	97.1	97.0	96.9			
	6	0.3	0.2	0.4	0.1	0.1	100	99.2	99.4	99.5	99.4	99.3	99.2	99.1	99.0	98.9	98.8	98.7	98.6	98.5	98.4	98.3	98.2	98.1	98.0	97.9	97.8	97.7	97.6	97.5	97.4	97.3	97.2	97.1	97.0		
	7	0.4	0.3	0.5	0.2	0.3	0.2	100	99.0	99.1	99.2	99.1	99.0	98.9	98.8	98.7	98.6	98.5	98.4	98.3	98.2	98.1	98.0	97.9	97.8	97.7	97.6	97.5	97.4	97.3	97.2	97.1	97.0	96.9	96.8		
	8	0.5	0.4	0.6	0.3	0.4	0.3	0.2	100	98.9	99.0	99.1	99.0	98.9	98.8	98.7	98.6	98.5	98.4	98.3	98.2	98.1	98.0	97.9	97.8	97.7	97.6	97.5	97.4	97.3	97.2	97.1	97.0	96.9	96.8		
	9	0.5	0.4	0.6	0.3	0.4	0.3	0.2	0.1	100	98.8	98.9	99.0	98.9	98.8	98.7	98.6	98.5	98.4	98.3	98.2	98.1	98.0	97.9	97.8	97.7	97.6	97.5	97.4	97.3	97.2	97.1	97.0	96.9	96.8		
	10	0.4	0.3	0.5	0.2	0.3	0.2	0.1	0.1	0.1	100	98.7	98.8	98.9	98.8	98.7	98.6	98.5	98.4	98.3	98.2	98.1	98.0	97.9	97.8	97.7	97.6	97.5	97.4	97.3	97.2	97.1	97.0	96.9	96.8		
	11	0.5	0.4	0.6	0.3	0.4	0.3	0.2	0.1	0.1	0.1	100	98.6	98.7	98.8	98.7	98.6	98.5	98.4	98.3	98.2	98.1	98.0	97.9	97.8	97.7	97.6	97.5	97.4	97.3	97.2	97.1	97.0	96.9	96.8		
	12	0.5	0.4	0.6	0.3	0.4	0.3	0.2	0.1	0.1	0.1	0.1	100	98.5	98.6	98.7	98.6	98.5	98.4	98.3	98.2	98.1	98.0	97.9	97.8	97.7	97.6	97.5	97.4	97.3	97.2	97.1	97.0	96.9	96.8		
	13	0.5	0.4	0.6	0.3	0.4	0.3	0.2	0.1	0.1	0.1	0.1	0.1	100	98.4	98.5	98.6	98.5	98.4	98.3	98.2	98.1	98.0	97.9	97.8	97.7	97.6	97.5	97.4	97.3	97.2	97.1	97.0	96.9	96.8		
	14	0.5	0.4	0.6	0.3	0.4	0.3	0.2	0.1	0.1	0.1	0.1	0.1	0.1	100	98.3	98.4	98.5	98.4	98.3	98.2	98.1	98.0	97.9	97.8	97.7	97.6	97.5	97.4	97.3	97.2	97.1	97.0	96.9	96.8		
	15	0.5	0.4	0.6	0.3	0.4	0.3	0.2	0.1	0.1	0.1	0.1	0.1	0.1	0.1	100	98.2	98.3	98.4	98.3	98.2	98.1	98.0	97.9	97.8	97.7	97.6	97.5	97.4	97.3	97.2	97.1	97.0	96.9	96.8		
	16	0.5	0.4	0.6	0.3	0.4	0.3	0.2	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	100	98.1	98.2	98.3	98.2	98.1	98.0	97.9	97.8	97.7	97.6	97.5	97.4	97.3	97.2	97.1	97.0	96.9	96.8		
	17	0.5	0.4	0.6	0.3	0.4	0.3	0.2	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	100	98.0	98.1	98.2	98.1	98.0	97.9	97.8	97.7	97.6	97.5	97.4	97.3	97.2	97.1	97.0	96.9	96.8		
	18	0.5	0.4	0.6	0.3	0.4	0.3	0.2	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	100	97.9	98.0	98.1	98.0	97.9	97.8	97.7	97.6	97.5	97.4	97.3	97.2	97.1	97.0	96.9	96.8		
	19	0.5	0.4	0.6	0.3	0.4	0.3	0.2	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	100	97.8	97.9	98.0	97.9	97.8	97.7	97.6	97.5	97.4	97.3	97.2	97.1	97.0	96.9	96.8		
	20	0.5	0.4	0.6	0.3	0.4	0.3	0.2	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	100	97.7	97.8	97.9	97.8	97.7	97.6	97.5	97.4	97.3	97.2	97.1	97.0	96.9	96.8		
	21	0.5	0.4	0.6	0.3	0.4	0.3	0.2	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	100	97.6	97.7	97.8	97.7	97.6	97.5	97.4	97.3	97.2	97.1	97.0	96.9	96.8		
	22	0.5	0.4	0.6	0.3	0.4	0.3	0.2	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	100	97.5	97.6	97.7	97.6	97.5	97.4	97.3	97.2	97.1	97.0	96.9	96.8		
	23	0.5	0.4	0.6	0.3	0.4	0.3	0.2	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	100	97.4	97.5	97.6	97.5	97.4	97.3	97.2	97.1	97.0	96.9	96.8		
	24	0.5	0.4	0.6	0.3	0.4	0.3	0.2	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	100	97.3	97.4	97.5	97.4	97.3	97.2	97.1	97.0	96.9	96.8		
	25	0.5	0.4	0.6	0.3	0.4	0.3	0.2	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	100	97.2	97.3	97.4	97.3	97.2	97.1	97.0	96.9	96.8		
	26	0.5	0.4	0.6	0.3	0.4	0.3	0.2	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	100	97.1	97.2	97.3	97.2	97.1	97.0	96.9	96.8	
	27	0.5	0.4	0.6	0.3	0.4	0.3	0.2	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	100	97.0	97.1	97.2	97.1	97.0	96.9	96.8
	28	0.5	0.4	0.6	0.3	0.4	0.3	0.2	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	100	96.9	97.0	97.1	97.0	96.9

**Fig. 6:** Details of 6 RHDV-2 isolates and 22 sequences of rabbit hemorrhagic disease viruses obtained from GenBank and identities to other rabbit hemorrhagic disease virus strains (isolates GenBank accession numbers: OQ925947 Assiut .vac1, OQ925948 Assiut .vac2, OQ925949 Assiut .vac3, OQ925950 Assiut .vac4, OQ925951 Assiut .vac5, and OQ925952 Assiut .vac6

Maximum Parsimony analyses produced the phylogenetic tree shown in (Fig.7)

containing the six RHDV strains and 45 reference strains.



**III. Pathogenicity of the isolated RHDV:**

**i. Clinical Signs**

The inoculated rabbits expressed RHDV-typical signs. anorexia, rapid respiration, cyanosis of lips and nostrils, bloody nasal discharge, and convulsions, along with other neurological symptoms like ataxia and paddling with legs near death. Ninety percent (90 %) mortality rate was recorded within 3–6 days post-infection. In a few cases, dead rabbits were found in the opisthotonus position exhibiting a backward arching of the head, neck, and spine due to muscle spasms

(Fig. 8). Additionally, mucoid fecal discharge with relaxed anal sphincter appeared.

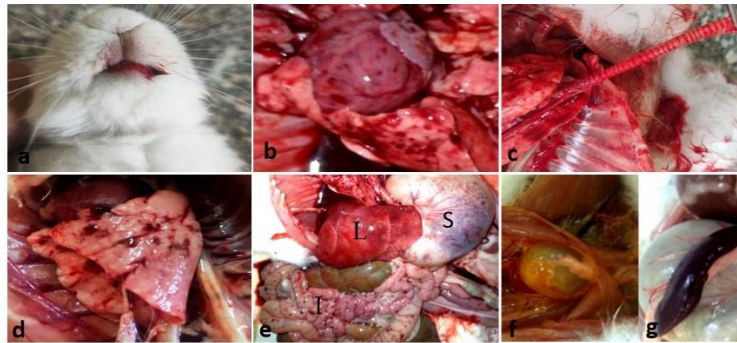
**ii. PM findings**

The most consistent lesion during P/M examination was hemorrhage almost in all organs (Fig. 8). The most severely affected organ was the liver (brownish and friable) while in weaning rabbits, the liver sometimes appeared to be pale with icteric discoloration. Trachea was often full of a foamy bloody exudate, lungs showed congestion, edema with multifocal punctuate hemorrhages of variable sizes accompanied by sub-pleural



hemorrhages, the spleen was swollen, severely congested and enlarged 2-3 times with rounded edges, kidneys showed hyperemic dark brown color and enlarged,

and urinary bladder was found full with turbid urine.

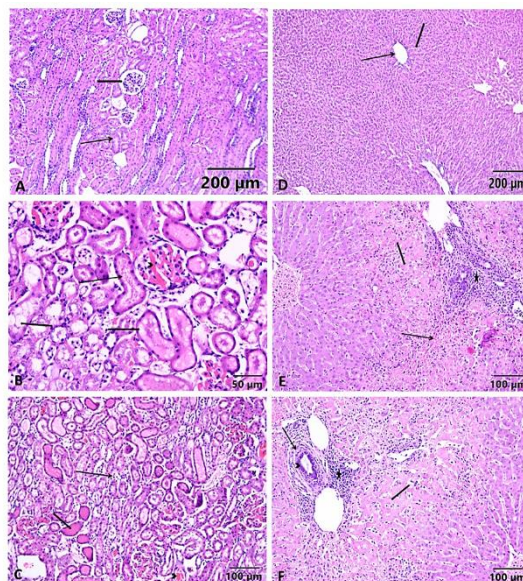


**Fig. 8:** RHDV experimentally infected rabbits showing: **a.** nasal and oral bloody expectoration, **b.** hyperemic coronaries of the heart with Focal ecchymotic epicardial hemorrhages, **c.** congested trachea with bloody exudate. **d.** congested lung with petechial and ecchymotic hemorrhages. **e.** enlarged liver (L) has a pale reticular pattern of necrosis and hemorrhages, and hyperaemic stomach (S) and intestine (I) with petechiae. **f.** distended urinary bladder with discoloured urine. **g.** enlarged spleen with black discoloration.

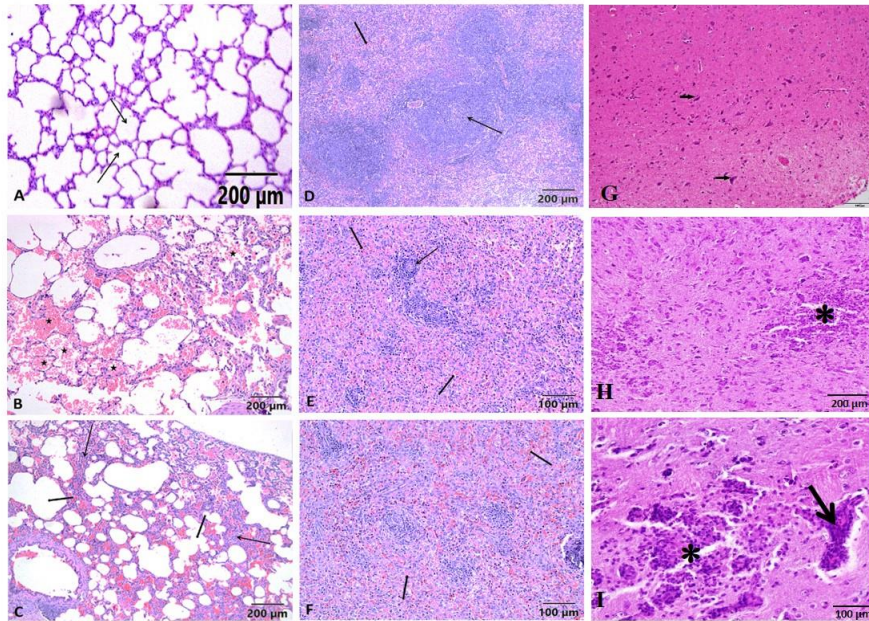
### iii. Histopathological changes

The infected rabbits presented scattered and variable histopathological changes in all

examined organs, liver, kidneys, lung, spleen, and brain. The changes are shown in (fig.9 and 10)



**Fig. 9:** Histopathological changes of rabbits infected with RHDV. **Kidney**, Control rabbits showing (A) Normal glomeruli (notched arrow) and normal renal tubules (arrow). Infected rabbits showing (B) Congestion of the glomerular capillary tufts (arrow head), coagulative necrosis of the cortical renal tubules with sloughing of the epithelium, pyknosis and karyolysis of the nucleus (arrow) and vacuolar degeneration of renal tubular epithelium (notched arrow). (C) Hyaline cast in renal tubular lumen (notched arrow) and interstitial infiltration of inflammatory cells (arrow). **Liver**, Control rabbits showing (D) Normal central vein (arrow) and normal cords of hepatocytes (notched arrow). Infected rabbits showing (E) Periportal necrosis of hepatocyte characterized by dissociation of hepatic cords, pyknosis and karyolysis of the nucleus and increase acidophilia of cytoplasm (notched arrow), hemorrhages of the liver (arrow) and infiltration of inflammatory cells in the portal area (star). (F) Periductal fibrosis (arrow), bile duct hyperplasia (arrow head), infiltration of inflammatory cells in the portal area (star) and periportal necrosis of hepatocyte (notched arrow).



**Fig. 10:** Histopathological changes of rabbits infected with RHDV. **Lung**, Control rabbits showing (A) Normal alveoli (arrow). Infected rabbits showing (B) Hemorrhages in the alveolar lumen (star). (C) Interstitial infiltration of inflammatory cells (arrow) and alveolar emphysema (notched arrow). **Spleen**, Control rabbits showing (D) Normal white pulp (arrow) and normal red pulp (notched arrow). Infected rabbits showing (E) Lymphocytic depletion in the white pulp (arrow) and hemorrhages in the red pulp (notched arrow). (F) Hemorrhages in the red pulp (notched arrow). **Brain**, G. normal cerebrum (normal microglia cell) (black arrowhead). H. Microglial proliferation (asterisk). I. Microglial nodule (asterisk) and perivascular cuffing (arrow). (H&E)

## DISCUSSION

Rabbit hemorrhagic disease is a contagious disease affecting domestic rabbits at different ages which is restricted by vaccination programs (Calvete *et al.*, 2018). In Egypt, RHDV outbreaks still occur in different lower and upper governorates causing significant mortality rates of notable economic losses during the last years despite the availability of RHDV vaccines (Magouz *et al.*, 2019). Nowadays, RHDV2 has gradually become the predominant strain in rabbit flocks (Desouky *et al.*, 2023).

This study involved isolation and detection of RHDV from 40 suspected RHDV pooled samples in 10 different Egyptian governorates between June 2022 and March 2023.

Unfortunately, the previous record indicated that RHV2 was the most predominant among

young ages but the current work isolated RHV2 from both adult and young rabbits. These finding could be supported by (Dalton *et al.*, 2012). This wide age range is indicative of RHV2's potential endemicity and that needs to be ascertained in large-scale studies. The examined rabbit flocks suffered from depression, conjunctivitis, nervous signs, frothy bloody nasal discharge, and friable pale liver with hemorrhages, accompanied by an enlarged congested hemorrhagic spleen and kidneys, as well as hemorrhagic tracheitis, congested edematous and hemorrhagic lungs. These findings confirmed the suspicions of RHDV infection as previously mentioned by numerous authors (El-Samadony *et al.*, 2021; Abodalal *et al.*, 2022, and Desouky *et al.*, 2023), these sever clinical signs are due to infection with the pathogenic strain of RHDV (Sahraoui *et al.*, 2023).

Cultivation of the virus in cell cultures were very difficult, therefore virus detection and

characterization were carried out by inoculation of susceptible rabbits (Ismail *et al.*, 2017).

The liver specimen was the main tissue collected from infected rabbits for viral identification, because it contains the highest viral titers and virus antigen was found in the liver already from 12h to 24h post infection, mainly in periportal areas. (Ahmad *et al.*, 2011 and Prieto *et al.* 2000).

HA test was be considered the first step of laboratory diagnosis of RHDV is using human type “O” and scientists of China and Europe used HA test as a screening test (Calvete *et al.*, 2002).

In this study, the molecular analysis identified RHDV in 16 cases and were hemagglutinin-positive with titers ranged from  $2^3$  to  $2^{14}$ . These results are nearly agreed with the study of Erfan and Shalaby (2020) who identified 16 cases that were positive for RHDV using PCR, and these 16 cases were hemagglutinin-positive, with titers ranging from  $2^9$  to  $2^{16}$ . Also these results are consistent with those reported previously (Le Gall-Recule *et al.*, 2013) that indicated that RHDV2 efficiently agglutinates human type O RBCs and confirmed the use of HA as a routine diagnostic tool for the detection of RHDV2 in infected samples. On the other hand Abd El-Moaty *et al.* (2014) observed that some isolates maybe non-Hemagglutinating and other may show hemagglutination after passaging in susceptible rabbits in Egypt. The same result were recorded by, Tian *et al.* (2007) observed isolates showed that there is no correlativity between the HA activity and the genetic typing of either classical or variant RHDV strains. So the HA test was not reliable for diagnosis or typing of RHDV field.

Nucleotide BLAST analysis in conjunction with VP60 sequencing were performed on the selected six isolates which revealed that six isolates were clustered with Egyptian RHDVb/2 strains. The nucleotide sequence

identities of the sequenced six isolates were 98.8-100 % compared to each other. 99.8% identities to MN276176vet-Abotaleb and 100% to other available RHDV2 strains (MW679028, MW455120GH-19, MW455122kfs-1-19, MW455123kfs-219, MW455124BH-K-19, MW455125Alex-19 were observed.

The recently isolated strains had nucleotide difference 23,1 % when compared to commonly vaccinal strain (JQ995154Giza 2006). This indicates that there is high genetic diversity between isolates and vaccinal strain. So, the continuous and rapid development of those RHD viruses necessitates reviewing and updating for vaccine development. The same result was recorded by (Hemida *et al.*, 2020).

Reviewing the available data by the author, our study is considered the first record about existence of RHDV2 infections among different rabbit ages in Upper Egypt

Our results agreed with previous record by Abodalal and Tahoon 2020; Erfan and Shalaby 2020; Desouky *et al.*, 2023 who isolated RHDV2 from the Lower Egypt. Our records could be attributed to the movement of some rabbit breeds from Lower Egypt to Upper Egypt by rabbit producer. This finding was supported by according to Calvete *et al.*, 2021 who found that surviving rabbits can act as virus carriers for several weeks. Thus, importation of apparently healthy rabbits might be a source of RHDV GI.2 to upper Egypt and, therefore, should be highly regulated.

The current work isolated RHDV2 from both vaccinated and non-vaccinated rabbits that confirm that there is no cross protection between RHDV1 and RHDV2 thus, we recommended the necessity for vaccination rabbits with bivalent vaccine. These results are supported by (OIE, 2021; Abodalal and Tahoon 2020).

Additionally the pathogenicity of RHDV revealed that the inoculated rabbits showed the identical clinical symptoms of natural RHD infection with death occurring 3–6 days post infection with mortality rate 90%. This results are coincided with Abodalal *et al.*, 2021 who reported that RHDV isolation in the inoculated rabbits revealed deaths in 3-5 days post-infection.

Regarding our Histopathological findings of the Liver showing Periportal necrosis of hepatocyte characterized by dissociation of hepatic cords, pyknosis and karyolysis of the nucleus and increase acidophilia of cytoplasm, haemorrhages of the liver and infiltration of inflammatory cells in the portal area, Periductal fibrosis and bile duct hyperplasia. The same liver changes were recorded by (Marcato *et al.*, 1991; Maddison and Mesquite, 2009; Carissa *et al.*, 2012; Duarte *et al.*, 2015; Lopes *et al.*, 2015; Mohamed *et al.*, 2022 and Wanting *et al.*, 2022).

Concerning the Lung lesions observed in our study, there were Hemorrhages in the alveolar lumen, interstitial infiltration of inflammatory cells, and alveolar emphysema. These findings could be supported by (Marcato *et al.*, 1991; Maddison and Mesquite, 2009; Duarte *et al.*, 2015 and Lopes *et al.*, 2015). On the other hand (Wanting *et al.*, 2022) reported that some alveolar spaces were narrowed and exhibited mild hemorrhages, while other alveolar spaces were filled and (Shijun *et al.*, 2020) observed that there was extensive hemosiderin deposition.

In our work, there were variable Kidney lesions as Congestion of the glomerular capillary tufts, coagulative necrosis of the cortical renal tubules with sloughing of the epithelium, pyknosis, and karyolysis of the nucleus. Vacuolar degeneration of renal tubular epithelium and Hyaline cast in renal tubular lumen and interstitial infiltration of inflammatory cells. Similar renal lesions were described by many authors (Carissa *et*

*al.*, 2012 and Shijun *et al.*, 2020). In contrast to Wanting *et al.*, 2022 found that there were no apparent abnormalities observed in the kidney.

The most significant histopathological lesions were found in the spleen showing Lymphocytic depletion in the white pulp and hemorrhages in the red pulp. These results are supported by Shijun *et al.*, (2020) and Wanting *et al.* (2022).

The most important microscopic lesion was observed in the brain showing microglial proliferation and perivascular cuffing. These findings were in agreement with (Xu *et al.*, 1985a; Xu *et al.*, 1985, and Carissa *et al.*, 2012).

The pathological changes are thought to be the result of a viremia with extensive circulatory dysfunction (Xu *et al.*, 1985a; Xu *et al.*, 1985b). So petechial hemorrhage and generalized congestion occur in almost all organs due to poor blood coagulation (Xu and Chen, 1988).

## CONCLUSION

In the current study, the presence of RHDV-2 strains was detected for the first time and confirmed threatening the rabbit population in some Upper Egypt provinces. Continuous monitoring and surveillance of the RHDV strains circulating in Egypt should be done. Complete genome sequences of VP60 of RHDV strains are essential to recognize any changes in the virus sequences and update the strain of vaccine. The present work isolated RHDV2 from both vaccinated and non-vaccinated rabbits. So we recommended applying the bivalent RHDV vaccine involving both RHDV-1 and RHDV-2 variant strains to protect against infection with both types because there is no cross-protection immunity between each other.

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## التعرف على معزولات مرض الفيروس النزفي في الأرنب المنتشرة حالياً

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لقد تزايد انتشار مرض الفيروس النزفي (RHDV) في الأرنب شاملاً الأرنب المحصنة في مصر حديثاً . لذلك أجريت الدراسة الحالية من أجل التعرف على معزولات فيروس RHDV في الأرنب المحلية المحصنة والغير محصنه في بعض محافظات مصر ما بين ٢٠٢٢ إلى ٢٠٢٣. تم تجميع العينات من عدة قطعان أرنب تتراوح أعمارهم من شهرين إلى أربعة شهور مع ارتفاع معدل النفوق , الأعراض المرضية, آفات ما بعد النفوق المتعلقة بمرض النزف الدموي للأرنب (RHDV). تم إجراء اختبار التلازن الدموي (HA) والتوصيف الجزيئي باستخدام النسخ العكسي الاستردادي لتفاعل البلمرة المتسلسل (RT-PCR) الذي يستهدف المنطقة الجينية المشفرة لبروتين الغلاف الجزئ VP60 وتحليل التسلسل ودراسات النشوء والتطور للتعرف على معزولات مرض النزفي الدموي الفيروسي في الأرنب المنتشرة حالياً وتصنيفها

أظهرت النتيجة أن ١٦ حالة من أصل ٤٠ حالة كانت ايجابية لفيروس الأرنب النزفي باستخدام متفاعلة البوليميرز المتسلسل وكانت هذه الحالات ايجابية للاختبار التلازن الدموي بمعيار يتراوح ما بين 214 to 23.

تم إجراء تسلسل النيكلوتيدات ودراسات النشوء والتطور على ستة معزولات وأسفرت النتيجة على أن جميع المعزولات الستة كانت من سلالات RHDV-2 والتي لأول مرة تسجل في صعيد مصر وسجلت على بنك الجينات بالأسماء التالية ( Assiut.vac1 -OQ925947 to Assiut.vac6- OQ925952 )

كانت هويات تسلسل النيوكليوتيدات للمعزولات الستة المتسلسلة ٩٨,٨-١٠٠% مقارنة ببعضها البعض. السلالات المعزولة حديثاً لديها اختلاف في النيوكليوتيدات بنسبة ٢٣,١% بالمقارنة مع سلالات اللقاحات الشائعة (JQ995154Giza 2006). أبدت الأرنب الملقحة علامات نموذجية لفيروس RHDV وافات ما بعد الوفاة وتم تسجيل معدل وفيات بنسبة تسعين بالمائة (٩٠%). خلال ٣ إلى ٦ أيام بعد الإصابة .

أظهر الفحص المجهرى وجود التهاب الكبد الناخر الحاد واحتقان ونزيف واسع الانتشار في جميع الأعضاء الداخلية. الكبد والرئتين والطحال والكلى والدماغ.