

Isolation and Molecular Characterization of Rabbit Haemorrhagic Disease Virus Strains Circulating in Rabbit Population Using Sequencing and Phylogenetic Analysis in Upper Egypt

Samah El Sayed Ali Abodalal¹, Mohammed Shaker Abdel Hafez², Eman Abd El-Munem Shosha^{3*}, Fatma Fadal Warda⁴, and Naglaa Mohammed Hagag⁵

¹Newcastle Disease Department, Veterinary Serum and Vaccine Research Institute, Agriculture research center, Abbasia, Cairo, Egypt, P.O.B.131

²Birds and Rabbits Diseases Department, Faculty of Veterinary Medicine, New Valley University, Egypt

³Microbiology and Immunology Department, Faculty of Veterinary Medicine, New Valley University, Egypt

⁴Horse sickness Research Department, Veterinary Serum and Vaccine Research Institute, Agriculture research center, Abbassia, Cairo, Egypt, P.O.B.131

⁵National Laboratory for Veterinary Quality Control on poultry production, AHRI, Agriculture Research Center P.O.Box264, Dokki, Giza

*Corresponding author's Email: emanshosha25@gmail.com; ORCID: 0000-0001-5862-3137

Received: 23 June 2021

Accepted: 01 September 2021

ABSTRACT

Rabbit hemorrhagic disease (RHD) is a contagious viral disease that threatens rabbit farms locally and globally. The disease causative agent is the RHD virus (RHDV) of the family *Caliciviridae*. The present study aimed to identify and characterize RHDV strains currently circulating in Upper Egypt provinces. A total of 20 suspected RHDV samples were collected from non-vaccinated rabbit flocks from January to December 2019 in Upper Egypt governorates (New Valley and Assuit), Egypt. The RHDV was confirmed through the hemagglutination test (HA) and reverse transcription-polymerase chain reaction (RT-PCR). Further characterization of selected 4 isolates was performed by nucleotide sequencing of a partial VP60 gene. All of 11 RHDV RT-PCR-positive samples were positive for HA activity against human RBCs type "O". Based on the nucleotide sequencing, the selected 4 isolates were clustered as RHDV-1 variant strains (G3-G5). The nucleotide sequence identities of the 4 isolates were 94.2-100 %, compared to available RHDV strains from GenBank. In conclusion, the presence of RHDV-1 variant strains was detected and confirmed that threatens the rabbit's populations in New Valley and Assuit governorates.

Keywords: Upper Egypt, Nucleotide sequencing, Rabbit hemorrhagic disease virus, Reverse transcription-polymerase chain reaction, VP60

INTRODUCTION

Rabbit hemorrhagic disease (RHD) is a rapidly fatal viral disease which remains a threat to rabbit farms worldwide (Dalton et al., 2015). It causes high mortality large economic losses in the rabbit industry. Rabbit hemorrhagic disease was first recorded clinically in China (XU, 1991) then it became quickly endemic through Asia and Europe (Alda et al., 2010; Abrantes et al., 2012). In Egypt, the rabbit hemorrhagic disease virus (RHDV) was firstly reported in the Sharkia governorate in 1991, and it was spread to other Egyptian governorates (Ghanem and Ismail, 1992; Hemida et al., 2020). Transmission of RHDV occurred through oral, conjunctival, nasal, and vector-like insect routes (Urakova et al., 2019). RHD causes severe petechial hemorrhages in multiple systemic organs, such as liver, trachea, and lungs (OIE, 2018). It was diagnosed by a hemagglutination (HA) test using

human-type "O" red blood cells (RBC). As there are non-hemagglutinating RHDV isolates, the HA test is unreliable for diagnosis (Bazid et al., 2015). Thus, virus detection and characterization are carried out through rabbits inoculation, reverse transcriptase-polymerase chain reaction (RT-PCR) (Ismail et al., 2017), and gene sequencing which facilitate all vaccine and wild-type virus strains to be fully identified and differentiated (Le Gall-Reculé et al., 2017; Kwit and Rzeżutka, 2019). The Egyptian authorities' control strategy of RHD depends mostly on rabbit vaccination with appropriate commercial vaccines (Abido et al., 2020).

Rabbit hemorrhagic disease virus is a single-stranded Ribonucleic acid positive-sense (ssRNA⁺) virus, non-enveloped classified within the family *Caliciviridae*, genus *Lagovirus* (Abrantes et al., 2012). This species would be divided into two genogroups that correspond to RHDV

(GI) and European Brown Hare Syndrome Virus (EBHSV) (GII) related viruses. Then, genogroups of RHDV strains could be subdivided into GI.1a/RHDVa for RHDVa (G6) strains, GI.1b/RHDV for classical RHDV G1, and GI.1c/RHDV for classical G2 strains. Furthermore, GI.1d/RHDV was proposed for the three classical genotypes G3/G4/G5. The recently described RHDV2 has a new proposed name GI.2/RHDV2/b (Le Pendu et al., 2017)

The RHDVa variant strain was identified in 2006 which substituted the classic RHDV strain in vaccine manufacture (Salman, 2007). The newly emerging RHDV2 caused various outbreaks in the vaccinated rabbits' flocks (Hemida et al., 2020) with a variable mortality rate; death can happen in adult and lactating rabbits from 15 days of age. (Le Gall-Reculé et al., 2013). Both classical and variant strains combination have resulted in enlarged diversity in RHDV strains (Lopes et al., 2018). RHDV and RHDV2 are identical in their genomic structures in which both contain two open reading frames (ORFs). ORF1 encodes the RNA-dependent RNA polymerase and the main capsid protein (VP60) and ORF2 encodes a minor structural protein known as VP10 (Dalton et al., 2015).

The VP60 is the major structural protein of RHDV capsid and it is the most immunogenic protein (Awad and Kotb, 2018). It consists of a buried shell (S) domain (N-terminus) and the protruding (P) domain (C-terminus) which is exposed to the surface. The P domain can be subdivided into two subdomains (P1 and P2) where P2 displays the greatest genetic variation (Neill, 1992; Abrantes et al., 2012). Six distinct regions (A-F) can be

discriminated against VP60 gene although C and E are located in the exposed P2 subdomain that shows the highest genetic variation (Puggioni et al., 2013).

In Egypt, severe mortalities were reported among vaccinated rabbit farms during 2018-2019 and the samples were RHDV positive from different governorates. Suspected cases were confirmed to be RHDV positive from different Lower Egyptian governorates (Abido et al., 2020; Erfan and Shalaby, 2020). This study was performed to investigate isolation and molecular characterization of RHDV strains circulating in rabbit population in New Valley and Assuit governorates using sequencing and phylogenetic analysis to know the emergence of RHDV2 in Upper Egypt provinces as Lower Egypt or not.

MATERIALS AND METHODS

Ethical approval

Institutional Animal Care and Use Committee at Veterinary Serum and Vaccine Research Institute for Evaluation of Veterinary Biologics acknowledge the research manuscript as it was reviewed under the current research authority and it was deemed compliance with bioethical standards in good faith.

Case history

Complete data about the investigated rabbitries were collected during the suspected RHDV outbreaks (Table 1). Rabbits in 11 intensive rabbit production farms representing two Upper Egyptian governorates (Table 1) exhibiting symptoms and lesions suspected to be RHDV from January to December 2019 with no history of vaccination against RHDV.

Table 1. Case history of the 11 investigated rabbitries (not vaccinated) suffered from rabbit hemorrhagic disease virus outbreaks from January to December 2019 in New Valley and Assuit governorates, Egypt

Farm number	Date	Farm capacity/ dams	Governorate	Mortality (%)			
				Suckling*	Weaning**	Growing***	Adult****
1	16/1/2019	70	New Valley	5	10	80	90
2	21/1/2019	90	Assuit	10	10	85	85
3	8/2/2019	100	Assuit	7	15	90	87
4	30/5/2019	50	New Valley	8	10	85	85
5.	20/8/2019	80	New Valley	5	18	90	80
6	18/8/2019	90	Assuit	7	20	85	90
7	14/5/2019	100	Assuit	8	10	80	90
8	23/6/2019	60	Assuit	6	15	87	90
9	20/7/2019	75	New Valley	10	12	86	90
10	8/12/2019	90	Assuit	5	10	90	85
11	15/12/2019	100	Assuit	6	20	80	90

*: Suckling rabbits aged 17-35 days, **: Weaning rabbits aged 35-55 days, ***: Growing rabbits aged 55 days up to 4 months, ****: Adult rabbit aged more than 4 months.

The clinical examination

The investigated rabbitries were examined for clinical signs during the suspected RHDV outbreak from January to December 2019 in Assuit and New valley

governorates, Egypt. Clinical signs noticed on the affected rabbits included pyrexia, cyanosis of lips and nostrils, hemorrhagic nasal discharges, ataxia, and convulsions.

Postmortem examination

The freshly dead rabbits during the suspected RHDV outbreaks were subjected to postmortem P/M examination with the recording of the observed macroscopic pathological findings.

Samples collection and preparation

Liver tissues were aseptically collected from freshly dead rabbits from different localities in Upper Egyptian governorates. Liver extract prepared after homogenization of 10% of liver tissue samples in phosphate buffer saline (PBS) weight per volume (w/v). The prepared suspensions were centrifuged at 3000 rpm for 15 minutes with chilling at 4°C (OIE, 2018). The clear supernatants were collected and kept at -20°C till used.

Haemagglutination test

Washed erythrocytes human-type "O" suspended in sterile saline as 0.75% and 10% for micro-hemagglutination (HA) technique and rapid slide HA tests, respectively. Two-fold dilutions of homogenized liver tissue suspension (10% w/v) with PBS were incubated with an equal volume of washed human RBCs type "O" (0.75% concentration) in a V shaped-bottom microtiter plate at 4°C according to a study conducted by Capucci *et al.* (1996a,b).

Isolation of rabbit hemorrhagic disease virus

Isolation was performed in the susceptible rabbits as reported by Capucci *et al.* (1991) who obtained a significant yield of highly purified RHDV from the liver of affected rabbits as the isolated virus reproduced the disease in susceptible rabbits and has been re-isolated from dead ones. Liver extracts from the freshly dead rabbits during RHDV outbreak in some Egyptian governorates were inoculated [1 ml/rabbit intramuscular (I/M)] into five susceptible crossbreed rabbits (aging 1 month old and seronegative for RHDV HI antibodies). Another five rabbits were inoculated with 1 ml sterile saline solution and kept as the negative control. All rabbits were kept under daily observation for two weeks with the recording of clinical signs, mortalities, and P.M. lesions. Liver extracts prepared from freshly dead rabbits for re-detection of RHDV through micro-HA test using human-type "O" RBCs.

RNA extraction

RNA extraction from the clarified liver tissue homogenates was performed according to Abd El-Moaty *et al.* (2014) using the QIAamp viral RNA Mini kit

(Qiagen, Gmbh, Germany) according to the manufacturer's instructions. Briefly, 140 µl of liver homogenates were incubated with 560 µl of AVL lysis buffer and 5.6 µl of carrier RNA at room temperature for 10 minutes. After complete lysis, 560 µl of ethanol of a concentration of 100% was added and mixed for 15 seconds by pulse vortexing. Aliquots of 630 µL were transferred to a spin column and centrifuged at 8000 rpm for one minute. The sample was washed in 500 µL AW1 buffer then centrifuged at 8000 rpm for one minute followed by adding 500 µL AW2 buffer then centrifuged again at 14,000 rpm for 3 minutes. The RNAs were eluted with 60 µl of elution buffer and stored at -80°C until used.

Detection of rabbit hemorrhagic disease virus by RT-PCR

PCR oligonucleotide primers (Metabion, Germany) that were designed according to Fahmy *et al.* (2010); to amplify 538 bp of the highly variable region of *VP60* gene Forward primer (P33): 5'CCACCACCAACTTCAGGT'3 and reverse primer (P34): 5' CAGGTTGAACACGAGTGTGC'3). The master mix was used in a total volume of 25 µl containing 12.5 µl of the 2x QuantiTect SYBR Green PCR Master Mix (Qiagen, Gmbh, Germany), 1 µl of each primer at a concentration of 20 pmol, 0.25 µl of Revert Aid reverse transcriptase, 7.25 µl of nuclease-free water, and 3 µl of RNA template. The reaction was performed in a BIO-RAD® PCR system T100 thermocycler (BioRad, Hercules, California, USA). Reverse transcription was carried out at 50°C for 30 minutes followed by a primary denaturation step at 95°C for 15 minutes, 40 cycles at 94°C for 55 seconds, 56°C for 55 seconds, and 72°C for 1 minute. A final extension step was performed at 72°C for 10 minutes.

Sequencing and phylogenetic analysis

The amplified *VP60* PCR products (15µl) were evaluated by gel electrophoresis using ultrapure 1.5% agarose (Invitrogen, Thermo Fisher Scientific, Germany) in 1×Tris-borate-EDTA (TBE) buffer at room temperature. Gelpilot 100 bp DNA ladder (Qiagen, Gmbh, Germany) was used to determine the product size. PCR-amplified bands were detected by imaging using a gel documentation system (Alpha Innotech, Biometra). Finally, data were analyzed using Automatic Image Capture Software (Protein Simple, formerly Cell Biosciences, San Jose, CA, USA).

Gene sequencing and phylogenetic analysis of PCR products were purified using a QIAquick PCR Product extraction kit (Qiagen, Gmbh, Germany). Sequence

reactions were performed using a Bigdye Terminator V3.1 cycle sequencing kit (Perkin-Elmer), and purification was carried out by Centri-Sep spin columns (Thermo Fisher, Germany). The *VP60* sequences were obtained using a 3500xl genetic analyzer (Applied Bio-systems, Life technologies, Thermo Fisher, Germany). Basic Local Alignment Search Tool (BLAST®) (Altschul et al., 1990) was performed to establish sequence similarities to the sequences deposited in the GenBank database. The MegAlignmodule of Lasergene DNA-Star version 12.1 was used to determine phylogenetic distances among the analyzed strains (Thompson et al., 1994) and MEGA7 was employed to create a phylogenetic tree using maximum composite likelihood with 1000 bootstrap replications, neighbor-joining, and maximum parsimony (Kumar et al., 2016) and assembled sequences were deposited to the GenBank database.

RESULTS

Case history

The investigation of eleven rabbitries suffered from high mortalities from January to December 2019 in some Upper Egypt governorates revealed that the RHDV outbreaks were distributed mainly in New Valley, and Assuit governorates (Table 1). The diagnosis of suspected RHDV cases was determined considering clinical signs, postmortem lesions, HA activity, conventional RT-PCR, and sequencing of the RHDV *VP60* gene. All the ultimately diagnosed RHDV samples (RHDV-positive) were from unvaccinated flocks. All groups of the examined rabbitries included the neonates suckling rabbits of less than 1 month old, the weaned rabbits of 1-2 months old, growing rabbits of 2-3 months old, the premature rabbits of 3-5 months old, and finally adult rabbits.

The mortality rate in the investigated rabbitries ranged 5-10% in suckling rabbits aged 17-35 days. However, the mortality rate was reported at 80-90% in the adult rabbit aged more than 4 months. Furthermore, in growing rabbits aged 55 days up to 4 months, the mortalities were within the range of 80-90%, and in weaning rabbits aged 35-55 days, it was reported 10-20% (Table 1). Most of the examined rabbitries fed on the commercially formulated ration (pellet form). The housing system in the different examined rabbitries was wire cages.

Clinical features

Changeable clinical signs noticed on the affected rabbits in the examined rabbitries (Figure 1) included

pyrexia with increased respiratory rates as well as cyanosis of lips and nostrils, anorexia, hemorrhagic nasal discharges, and convulsions besides other neurological signs such as ataxia and paddling with legs that seemed approaching death. Occasionally, the dead rabbits were found in the opisthotonos position (spasm of the muscles causing backward arching of the head, neck, and spine). Moreover, the anal sphincter sometimes appears to loosen with mucoid fecal discharge.

P/M examination

The most consistent lesion during P/M examination was hemorrhaged almost in all organs accompanied by poor blood coagulation (Figure 1). 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 subpleural hemorrhages, the spleen was swollen, severely congested and enlarged 2-3 times with rounded edges, kidneys showed hyperaemic dark brown color and enlarged, and urinary bladder was found full with turbid urine.

Haemagglutination test

The 11 samples from RHDV RT-PCR-positive rabbits were also positive for HA activity against human RBCs type "O" in the microtiter plate. The HA titers varied from 2^9 to 2^{12} .

Isolation of rabbit hemorrhagic disease virus

RHDV was successfully isolated from different suspected RHDV outbreaks samples with the development of the specific and characteristic clinical signs as well as postmortem lesions for RHDV (Figure 1) in the inoculated rabbits. The deaths occurred within 3-5 days post-infection. Neither signs nor deaths were recorded in the negative control group. The RHDV was detected in liver extracts of dead rabbits individually in all RHDV outbreaks after isolation using microtiter plate HA test against human RBCs "O" type.

Molecular identification

All the examined RHDV samples (n = 11) from diseased rabbits were found to be positive for RHDV when tested by the conventional RT-PCR using *VP60* specific primers. The amplified *VP60* gene was successfully done as the anticipated amplicon size 538bp was clearly detected in all examined samples (Figure 2).

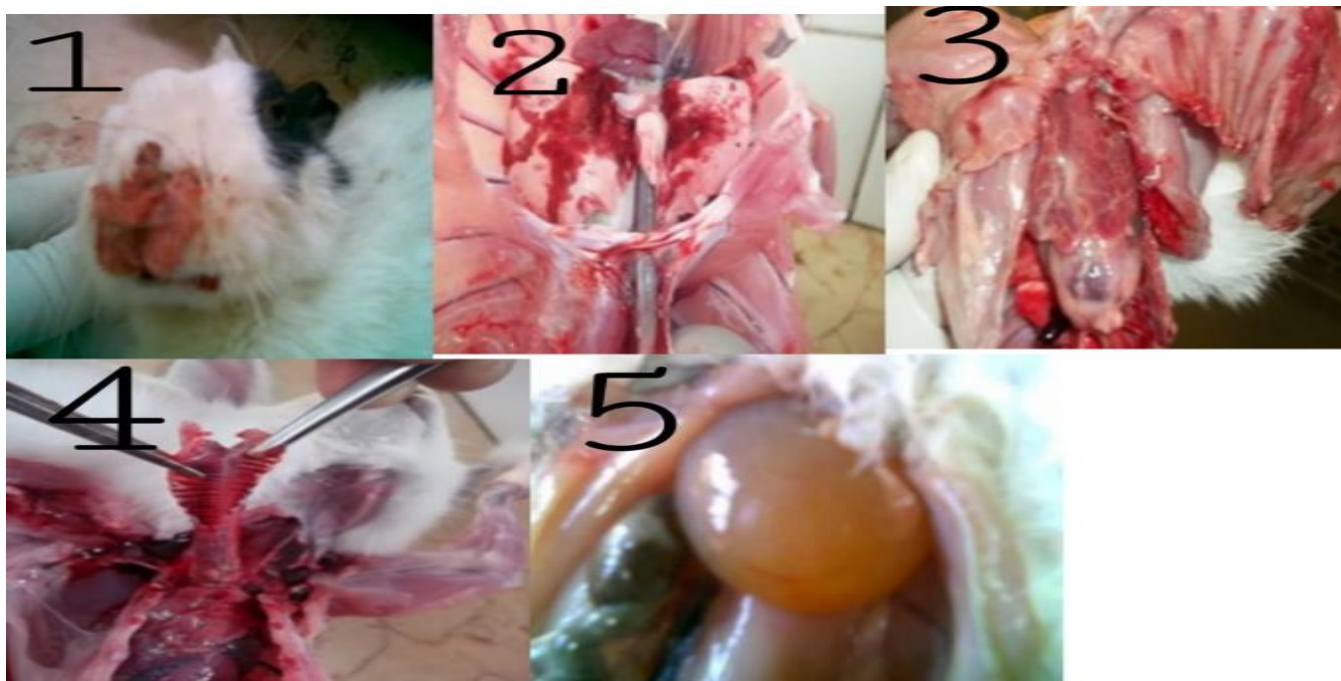


Figure 1. Clinical signs and postmortem lesions of suspected rabbit haemorrhagic disease virus outbreaks samples collected from Californian and Netherland rabbits in New Valley and Assuit governorates, Egypt in 2019. **1:** Haemorrhagic nasal discharges, **2:** Lungs are oedematous, congested, and hemorrhagic with splenomegaly, **3:** Liver appears yellowish-brown in color, brittle and degenerated with a marked lobular pattern, **4:** tracheal mucosa is hyperaemic and containing abundant frothy fluid, **5:** Urinary bladder engorged with discolored urine.

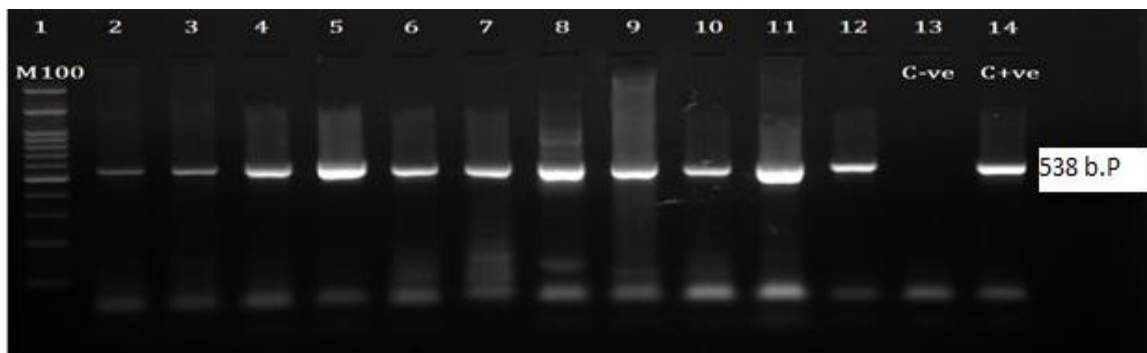


Figure 2. Detection of rabbit hemorrhagic disease virus using RT-PCR. Agarose-gel electrophoresis of amplified products of 538 bp of rabbit hemorrhagic disease virus using *VP60* specific primers. Lane **1:** 100bp, DNA size marker, lane **13:** Negative control, lane **2-12:** RHDV suspected tissue samples (positive), lane **14:** Positive control

Nucleotide sequencing and phylogenetic analysis

The phylogenetic tree was constructed by the Neighbor-joining method for the nucleotide sequence of RHDV for the highly variable region of *VP60* gene. Samples were carefully chosen from different localities of two Upper Egypt governorates. All of them were included for a sequence of the highly variable region of *VP60* gene (C-E region). The sequencing and phylogenetic analysis of *VP60* gene revealed that four isolates (RHD-1, RHD-2, RHD-4, and RHD-5) were closely related to RHDV-1 strains (G3-G5), compared to RHDV strains available

from GenBank (Figure 5). The nucleotide sequence identities of four isolates were 94.2-100 % compared to other available RHDV strains (Figure 4). On the other side, these isolates showed 100% nucleotide identity among them (Figure 2). The partial *VP60* (C-E region) sequences of four isolates were submitted to GenBank with accession numbers: MW251513, MW251514, MW251516, and MV251517. The alignment of 118 amino acids of RHDV-1 variant isolates and 29 RHDV sequences obtained from GenBank with their details listed in (Figure 3) was conducted.

Majority	MASGIISTPNANAIITYTPQDRIVTTPGTPAAAPVGNKNTPIMFASVVRRTGVDNASAGSTNGTQYGTGSQ	
		10 20 30 40 50 60 70
LT549473.1-E14-73-France-2014	...V...SS.....N...NA.....I.....I.E.....A...	208
LT168839.1-RHDV2-13-22-France-2013	...V...SS.....N...NA.....I.....I.E.....A...	208
KJ683895.1-RHDV2-Portugal-2013	...V...SS.T.....N...NA.....I.....I.E.....A...	208
MT067629-RHDV-A-Qalubia-Egypt-2019	...V...SS.T.....N...NA.....I.....I.E.....A...	208
MT067630-RHDV-B-Qalubia-Egypt-2019	...V...SS.T.....N...NA.....I.....I.E.....A...	208
AY269825.1-Isolate-NJ-China-1985	...V.....V.....S.....A.....	208
DQ069280.1-RHDV-whn-china-2005	...V.....V.....K.....A.....	208
FR823355.1-RHD-90-10	...V.....T.....A.....	208
Z24757.1-AST-89	...V...S.....T.....A.....	208
M67473-RHD-FRG-91-GER(G1)S.....T.....A.....	208
FN552800.1-RHDV-01-15-CYM74T.....	208
DQ189077.1-RHDV-BahrainT.....	208
AY925209.1-RHD-Ireland1	208
EF558572.1-RHDV-Frankfurt-12T...AS.....	208
EF222287.1-RHDV-Egypt-KS-2000AS.....	208
RHD1-Assuit-Egypt-2019	208
RHD2-Assuit-egypt-2019	208
RHD4-wadielgidid-Egypt-2019	208
RHD5-wadielgidid-Egypt-2019	208

Figure 3. Deduced amino acids alignment of *VP60* gene. Deduced amino acids of 538bp fragment (118 amino acids) of *VP60* gene of RHDV-1 isolates and 29 sequences of rabbit hemorrhagic disease viruses obtained from GenBank. The isolates belonging to RHDV-1 strains with 100% with RHD-Ireland1 with accession number AY925209.1. RHDV-1 identical amino acids are represented with dots (.) and letters represent mismatches using MEGAX and BioEdit software packages

		Percent Identity																				
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19		
Divergence	1	100.0	98.6	97.1	97.1	84.1	84.1	85.5	84.1	85.5	85.5	85.5	85.5	82.6	82.6	85.5	85.5	85.5	85.5	85.5	1	LT549473.1-E14-73-France-2014
	2	0.0	98.6	97.1	97.1	84.1	84.1	85.5	84.1	85.5	85.5	85.5	85.5	82.6	82.6	85.5	85.5	85.5	85.5	85.5	2	LT168839.1-RHDV2-13-22-France-2013
	3	1.5	1.5	98.6	98.6	84.1	84.1	84.1	82.6	84.1	84.1	84.1	84.1	81.2	81.2	84.1	84.1	84.1	84.1	84.1	3	KJ683895.1-RHDV2-Portugal-2013
	4	3.0	3.0	1.5	100.0	85.5	85.5	85.5	84.1	85.5	85.5	85.5	85.5	82.6	82.6	85.5	85.5	85.5	85.5	85.5	4	MT067629-RHDV-A-Qalubia-Egypt-2019
	5	3.0	3.0	1.5	0.0	85.5	85.5	85.5	84.1	85.5	85.5	85.5	85.5	82.6	82.6	85.5	85.5	85.5	85.5	85.5	5	MT067630-RHDV-B-Qalubia-Egypt-2019
	6	18.0	18.0	18.0	16.1	16.1	97.1	94.2	92.8	91.3	94.2	94.2	94.2	91.3	91.3	94.2	94.2	94.2	94.2	94.2	6	AY269825.1-Isolate-NJ-China-1985
	7	18.0	18.0	18.0	16.1	16.1	3.0	94.2	92.8	91.3	94.2	94.2	94.2	91.3	91.3	94.2	94.2	94.2	94.2	94.2	7	DQ069280.1-RHDV-whn-china-2005
	8	16.1	16.1	18.0	16.1	16.1	6.0	6.0	98.6	97.1	97.1	97.1	95.7	97.1	95.7	95.7	95.7	95.7	95.7	95.7	8	FR823355.1-RHD-90-10
	9	18.0	18.0	19.8	18.0	18.0	7.6	7.6	1.5	95.7	95.7	95.7	94.2	95.7	94.2	94.2	94.2	94.2	94.2	94.2	9	Z24757.1-AST-89
	10	16.1	16.1	18.0	16.1	16.1	9.3	9.3	3.0	4.5	97.1	97.1	95.7	97.1	95.7	95.7	95.7	95.7	95.7	95.7	10	M67473-RHD-FRG-91-GER(G1)
	11	16.1	16.1	18.0	16.1	16.1	6.0	6.0	3.0	4.5	3.0	100.0	98.6	97.1	95.7	98.6	98.6	98.6	98.6	98.6	11	FN552800.1-RHDV-01-15-CYM74
	12	16.1	16.1	18.0	16.1	16.1	6.0	6.0	3.0	4.5	3.0	0.0	98.6	97.1	95.7	98.6	98.6	98.6	98.6	98.6	12	DQ189077.1-RHDV-Bahrain
	13	16.1	16.1	18.0	16.1	16.1	6.0	6.0	4.5	6.0	4.5	1.5	1.5	95.7	97.1	100.0	100.0	100.0	100.0	100.0	13	AY925209.1-RHD-Ireland1
	14	19.8	19.8	21.8	19.8	19.8	9.3	9.3	3.0	4.5	3.0	3.0	3.0	4.5	98.6	95.7	95.7	95.7	95.7	95.7	14	EF558572.1-RHDV-Frankfurt-12
	15	19.8	19.8	21.8	19.8	19.8	9.3	9.3	4.5	6.0	4.5	4.5	4.5	3.0	1.5	97.1	97.1	97.1	97.1	97.1	15	EF222287.1-RHDV-Egypt-KS-2000
	16	16.1	16.1	18.0	16.1	16.1	6.0	6.0	4.5	6.0	4.5	1.5	1.5	0.0	4.5	3.0	100.0	100.0	100.0	100.0	16	RHD1-Assuit-Egypt-2019
	17	16.1	16.1	18.0	16.1	16.1	6.0	6.0	4.5	6.0	4.5	1.5	1.5	0.0	4.5	3.0	0.0	100.0	100.0	100.0	17	RHD2-Assuit-egypt-2019
	18	16.1	16.1	18.0	16.1	16.1	6.0	6.0	4.5	6.0	4.5	1.5	1.5	0.0	4.5	3.0	0.0	0.0	100.0	18	RHD4-Wadielgidid-Egypt-2019	
	19	16.1	16.1	18.0	16.1	16.1	6.0	6.0	4.5	6.0	4.5	1.5	1.5	0.0	4.5	3.0	0.0	0.0	0.0	19	RHD5-Wadielgidid-Egypt-2019	
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19			

Figure 4. Details of RHDV-1 isolates and 29 sequences of rabbit hemorrhagic disease viruses obtained from GenBank and identities to other rabbit hemorrhagic disease virus strains (isolates GenBank accession numbers: MW251513, MW251514, MW251516, and MV251517)

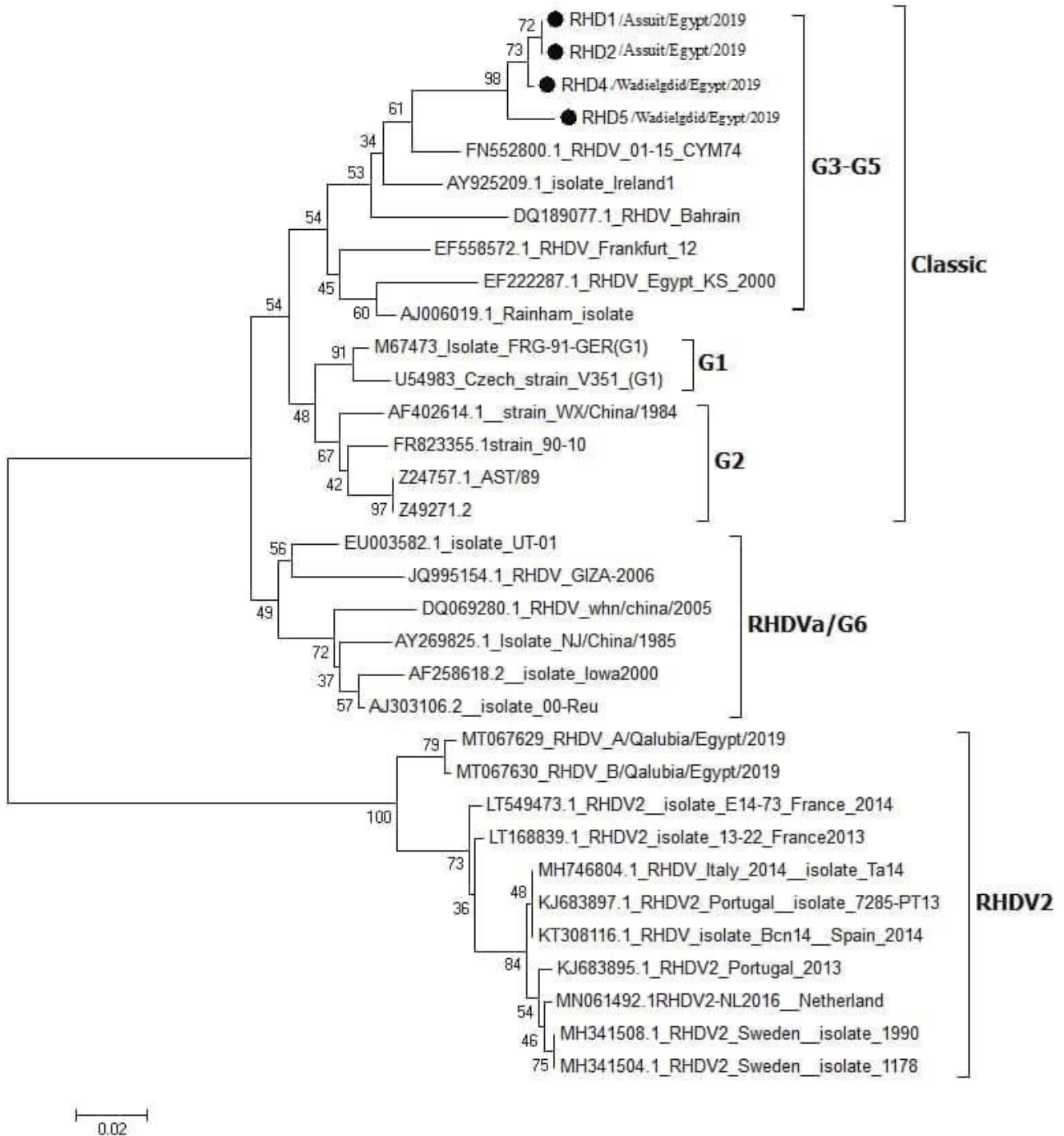


Figure 5. Phylogenetic tree of rabbit hemorrhagic disease virus based upon partial nucleotide sequences (*VP60* gene) and other randomly selected strains from GenBank (MEGA 6-Neighbor-joining). The circles show RHDV-1 variant isolates in different localities of two Upper Egypt governorates

DISCUSSION

Rabbit hemorrhagic disease is an important disorder of rabbit populations which restricted by vaccination programs. In Egypt, RHDV outbreaks still occur in

different governorates causing significant mortality rates of notable economic losses during the last years despite the availability of RHDV vaccines. RHDV was firstly reported in China in 1984 (Liu et al., 1984) then it became endemic in most European, Asian, and African countries

as well as in Australia and New Zealand (Grazioli et al., 2000).

In the current study, RHDV isolation in the inoculated rabbits revealed deaths in 3-5 days post-infection. Most of the investigated rabbitries were not vaccinated against RHDV. The examined rabbit farms showed high mortality rates (80-90 %) in adult rabbits (Table 1), these results agreed with (OIE, 2019) indicating higher mortality rates in adults 80-90% and subclinical form in rabbits younger than 6-8 weeks as a result of RHDV/RHDVa. the present findings were similar to those reported by Erfan and Shalaby (2020) in which the older rabbits were positive for classical RHDV with a mortality rate of 75%. The clinical signs detected in the affected farms were in accordance with those mentioned by Awad and Kotb (2018) and (OIE, 2019), including nervous, respiratory signs, apathy, and anorexia.

The liver is the best organ of choice for virus identification as it comprises the highest virus titer (OIE, 2018). The prepared liver extracts were examined using a microtiter plate HA test. The RHDV agglutinates human-type "O" RBCs and was confirmed by the HA test as a routine diagnostic method for detection of RHDV in the suspected samples. All 11 samples were positive with HA titers varied from 2^9 to 2^{12} . These results were consistent with those stated by Le Gall-Reculé et al. (2013) and Bazid et al. (2015) indicating that RHDV isolates agglutinated human RBC of type "O". The RT-PCR assay detected RNA of RHDV in lung and liver samples, all 11 samples of suspected RHDV were positive. Amplification was performed for a 538 bp fragment of *VP60* gene highly conserved region of RHDV variants. These results were consistent with those of Le Gall-Reculé et al. (2017) who stated that *VP60* was an efficient target for RT-PCR assays. In addition, RT-PCR results approved that *VP60* (C-E region) could detect all RHDV genotypes as conserved region (Embury-Hyatt et al., 2012), but with time benefit as the produced 600 bp fragment could be directly sequenced for genotyping of suspected samples, especially in case of RHDV with negative HA activity (Abd El-Moaty et al., 2014).

The phylogeny of four selected RHDV samples collected in 2019 from two Egyptian provinces, and sequencing of *VP60* -capsid gene resulted in the identification of RHDV-1 variant strains with nucleotide identities ranging from 94.2-100%, compared to available RHDV-1 strains in Genbank. This finding agreed with Erfan and Shalaby (2020) who reported that the preliminary identification of RHDV-1 variant strains was in Upper Egypt governorates, but RHDV-2 variants were

identified primarily among the Lower Egypt provinces. Moreover, these results were in agreement with those reported by Abido et al. (2020) claiming that RHDV-2 was detected in Delta governorates, Egypt. Furthermore, these findings were consistent with those mentioned by Mahar et al. (2018) who detected the presence of both circulating RHDV-1 and RHDV-2 strains. Moreover, Abd El-Moaty et al. (2020) showed that the classical (GI.1d/RHDV) and variant (GI.1a) genotypes are still co-circulating in the Egyptian rabbit populations.

the present epidemiological survey in Assuit and NewValley provinces showed no emergent of RHDV-2 which agreed with Erfan and Shalaby (2020) who reported that a significant distribution of RHDV strains of genotypes (G3-G5) associated with the RHDV-1 variant strains presented commonly in Upper Egypt, while RHDV-2 circulated in Lower Egypt. Conclusively, these findings confirmed that the RHDV-1 variant strains still presented in Egypt which comes in agreement with studies conducted by El-Bagoury et al. (2014), Bazid et al. (2015), Magouzi et al. (2019), and Awad and Kotb (2018) reporting the presence of RHDV genotypes (G3-5) in the Egyptian fields.

CONCLUSION

In the current study, the presence of RHDV-1 variant strains was detected and confirmed threatening the rabbit population in some Upper Egypt provinces. Continuous monitoring and molecular characterization of the RHDV strains circulating in Egypt should be implemented. Complete genome sequences of RHDV strains are required to identify any changes in the virus sequences and update the vaccine strain. As RHDV-2 variant was identified among the Lower Egypt province, it may spread to Upper Egypt causing an outbreak. Accordingly, further investigation of other Upper Egypt governorates should be done to confirm the presence of RHDV-2. Thus, these findings underscore the urgent need to apply the bivalent RHDV vaccine involving both RHDV-1 and RHDV-2 variant strains to protect against infection with both types as there is no cross-protection immunity between each other.

DECLARATION

Acknowledgments

The authors would like to express their appreciation to the Faculty of Veterinary Medicine, New Valley University, and Veterinary Serum and Vaccine Research

Institute, Egypt for their collaboration, and support during all procedures of this experimental research.

Competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Authors' contributions

Eman Abd El-Munem SHOSHA and M. ABD EL HAFEZ collected samples and designed this study. Eman Abd El-Munem SHOSHA, Samah El Sayed Abo-Dalal, Naglaa M. Hagag, Fatma F.Warda performed the experimental works. Eman Abd El-Munem SHOSHA and Samah El Sayed Abo-Dalal performed the analysis, acquisition, and interpretation of data. Eman Abd El-Munem SHOSHA, Samah, El Sayed Abo-Dalal, and M. ABD EL HAFEZ drafted, revised the manuscript, and approved the final manuscript.

Ethical considerations

All authors approved the final draft of the manuscript for publication. Ethical issues (including plagiarism, consent to publish, misconduct, data fabrication and/or falsification, double publication and/or submission, and redundancy) have been checked by the authors.

REFERENCES

- Abd El-Moaty DA, El-Bagoury GF, El-Zeedy SA, and Salman OG (2014). Egyptian non-hemagglutinating isolates of rabbit hemorrhagic disease virus can change to variable HA profile. *Benha Veterinaria Medical Journal*, 26(2): 71-83. Available at: <https://www.bvmj.bu.edu.eg/issues/26-2/8.pdf>
- Abd El-Moaty DAM, Abo-Dalal SEA, Salman OGA, Abdel-Wanees N, and Abbas AM (2020). Molecular and serological studies of Egyptian strains of rabbit haemorrhagic disease virus and their comparison with vaccine strains. *Review Scientific Technical Office International des Epizooties*, 39(3): 2. DOI: <https://www.doi.org/10.20506/rst.39.3.3195>
- Abido OY, Mahmoud MA, Nahed Y, Ayman HED, Aziza MA, and Ahmed AES (2020). Protective efficacy of an inactivated vaccine against rabbit hemorrhagic disease virus 2 prepared from a local isolate in Egypt. *VacciMonitor*, 29(3): 143-150. Available at: <https://www.redalyc.org/jatsRepo/2034/203464874007/html/index.html>
- Abrantes J, Van der Loo W, Le Pendu J, and Esteves PJ (2012). Rabbit haemorrhagic disease (RHD) and rabbit haemorrhagic disease virus (RHDV): A review. *Veterinary Research*, 43(1): 12. DOI: <https://www.doi.org/10.1186/1297-9716-43-12>
- Alda F, Gaitero T, Suarez M, Merchan T, Rocha G, and Doadrio I (2010). Evolutionary history and molecular epidemiology of rabbit haemorrhagic disease virus in the Iberian Peninsula and Western Europe. *BMC Ecology and Evolution*, 10(1): 347. DOI: <https://www.doi.org/10.1186/1471-2148-10-347>
- Altschul SF, Gish W, Miller W, Myers EW, and Lipman DJ (1990). Basic local alignment search tool. *Journal of Molecular Biology*, 215(3): 403-410. DOI: [https://www.doi.org/10.1016/S0022-2836\(05\)80360-2](https://www.doi.org/10.1016/S0022-2836(05)80360-2)
- Awad NF, and Kotb GK (2018). Genetic characterization of rabbit hemorrhagic disease virus from naturally infected rabbits in Sharkia Governorate, Egypt. *Journal of Virological Sciences*, 3(1): 10-19. Available at: <https://www.bibliomed.org/mnsfulltext/118/118-1509341160.pdf?1626096648>
- Bazid AH, AboElkhair MM, Abdel-Razik AG, Salim R, Sultan HA, and Hussein HA (2015). Molecular characterization of a hemagglutinating and non-hemagglutinating rabbit hemorrhagic disease virus from Egypt. *Alexandria Journal of Veterinary Science*, 45(1): 1-5. DOI: <https://www.doi.org/10.5455/ajvs.177331>
- Capucci L, Chasey D, Lavazza A, and Westcott D (1996a). Preliminary characterization of a non-haemagglutinating strain of rabbit haemorrhagic disease virus from the United Kingdom. *Journal of Veterinary Medicine*, 43: 245-250. DOI: <https://www.doi.org/10.1111/j.1439-0450.1996.tb00311.x>
- Capucci L, Fusi P, Lavazza A, Pacciarini ML, and Rossi C (1996b). Detection and preliminary characterization of a new rabbit calicivirus related to rabbit hemorrhagic disease virus but nonpathogenic. *Journal of Virology*, 70(12): 8614-8623. DOI: <https://www.doi.org/10.1128/JVI.70.12.8614-8623.1996>
- Capucci L, Scicluna MT, and Lavazza A (1991). Diagnosis of viral haemorrhagic disease of rabbits 415 and the European brown hare syndrome. *Revue Scientifique et Technique International Office of Epizootics*, 10: 347-370. DOI: <https://www.doi.org/10.20506/rst.10.2.561>
- Dalton KP, Abrantes J, Lopes AM, Nicieza I, Álvarez AL, Esteves PJ, and Parra F (2015). Complete genome sequence of two rabbit hemorrhagic disease virus variant b isolates detected on the Iberian Peninsula. *Archives of Virology*, 160(3): 877-881. DOI: <https://www.doi.org/10.1007/s00705-014-2329-3>
- El-Bagoury GF, Abd El-Moaty DAM, El-Zeedy SAR, El-Nahas EM, and Youssif AA (2014). Molecular identification of RHDV Egyptian strains based on the highly variable region of VP60 gene. *Benha Veterinary Medical Journal*, 26(2): 84-100. Available at: <https://bvmj.journals.ekb.eg>
- Embury-Hyatt C, Postey R, Hisanaga T, Lynn B, Hooper-McGrevy K, McIntyre L, Millar K, and Pasick J (2012). The first reported case of rabbit hemorrhagic disease in Canada, *The Canadian Veterinary Journal*, 53: 998-1002. Available at: <https://europepmc.org/article/med/23450867>
- Erfan AM, and Shalaby AG (2020). Genotyping of rabbit hemorrhagic disease virus detected in diseased rabbits in Egyptian Provinces by VP60 sequencing. *Veterinary World*, 13(6): 1098-1107. DOI: <https://www.doi.org/10.14202/vetworld.2020.1098-1107>
- Fahmy HA, Arafa A, and Mahmoud AH (2010). Molecular diagnosis of rabbit hemorrhagic disease virus (RHDV). *Egyptain Journal of Comparative Pathology and Clinical Pathology*, 23(1): 85-101. Available at: <http://erepository.cu.edu.eg/index.php/EJCPCP/article/view/276>
- Ghanem IA, and Ismail AN (1992). Occurrence of rabbit hemorrhagic disease in Sharkia province. *Zagzeg Veterinary Medicine Journal*, 20(4): 491-502. Available at: <http://erepository.cu.edu.eg/index.php/EJCPCP/article/view/276>
- Grazioli S, Agnoletti F, Scicluna MT, Masoero N, Guercio A, Fallacara F, Lavazza A, Brocchi E, and Capucci L (2000). Rabbit haemorrhagic disease virus (RHDV) subtype "A" (RHDVa) is replacing the original strain in some Italian regions. In: Brocchi E, Lavazza A (eds), *Fifth International Congress of the European Society for Veterinary Virology*, Brescia, Italy, pp. 202-203. Available at: <https://www.scienceopen.com/document?vid=a7a54b78-9e40-461a-90e2-ad34d727a1cb>
- Hemida RE, Khalil SA, Al-Ebshahy EM, and Abotaleb MM (2020). Comparative study between the isolated rabbit hemorrhagic septicemia virus and available vaccine strain. *International Journal*

- of Veterinary Science, 9(2): 189-195. DOI: <https://www.doi.org/10.37422/IJVS/20.004>
- Ismail MM, Mohamed MH, El-Sabagh IM, and Al-Hammadi MA (2017). Emergence of new virulent rabbit hemorrhagic disease virus strains in Saudi Arabia. *Tropical Animal Health and Production*, 49(2): 295-301. DOI: <https://www.doi.org/10.1007/s11250-016-1192-5>
- Kumar S, Stecher G, and Tamura K (2016). MEGA7: Molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Molecular Biology and Evolution*, 33: 1870-1874. DOI: <https://www.doi.org/10.1093/molbev/msw054>
- Kwit E, and Rzeżutka A (2019). Molecular methods in detection and epidemiologic studies of rabbit and hare. *Viruses: A review. Journal of Veterinary Diagnostic Investigation*, 31(4): 497-508. DOI: <https://www.doi.org/10.1177/1040638719852374>
- Le Gall-Reculé G, Lavazza A, Marchandeu S, Bertagnoli S, Zwengelstein F, Cavadini P, Martinelli N, Lombardi G, Guérin JL, Lemaitre E et al. (2013). Emergence of a new lagovirus related to rabbit haemorrhagic disease virus. *Veterinary Research*, 44(1): 81. DOI: <https://www.doi.org/10.1186/1297-9716-44-81>
- Le Gall-Reculé G, Lemaitre E, Bertagnoli S, Hubert C, Top S, Decors A, Marchandeu S, and Guitton JS (2017). Large-scale lagoon disease outbreaks in European brown hares (*Lepus europaeus*) in France caused by RHDV2 strains spatially shared with rabbits (*Oryctolagus cuniculus*). *Veterinary Research*, 48(1): 70. DOI: <https://www.doi.org/10.1186/s13567-017-0473-y>
- Le Pendu J, Abrantes J, Bertagnoli S, Guitton JS, Le Gall-Reculé G, Lopes AM, Marchandeu S, Alda F, and Almeida T (2017). Proposal for a unified classification 483 system and nomenclature of lagoviruses. *Journal of General Virology*, 98: 1658-1666. Available at: <https://www.sciencedirect.com/science/article/pii/S1567134820301416>
- Liu SJ, Xue HP, and Pu BQ (1984). New viral disease in rabbits. *Animal Husbandry and Veterinary Medicine*, 16: 253-255. Available at: <https://www.cabdirect.org/cabdirect/abstract/19852264426>
- Lopes AM, Blanco-Aguilar J, Martín-Alonso A, Leitao M, Foronda P, Mendes M, Gonçalves D, Abrantes J, and Esteves PJ (2018). Full genome sequences are key to disclose RHDV2 emergence in the Macaronesian Islands. *Virus Genes*, 54(1): 1-4. DOI: <https://www.doi.org/10.1007/s11262-017-1523-2>
- Magouzi AF, Elsayedi EA, and Metwally AY (2019). Detection and characterization of rabbit haemorrhagic diseases virus strains circulating in Egypt. *Bulgarian Journal of Veterinary Medicine*, 22(4): 409-418. DOI: <https://www.doi.org/10.15547/bjvm.2085>
- Mahar JE, Hall RN, Peacock D, Kovaliski J, Piper M, Mourant R, Huang NN, Campbell S, Gu XN, Read A et al. (2018). Rabbit hemorrhagic disease virus 2 (RHDV2; GL2) is replacing endemic strains of RHDV in the Australian landscape within 18 months of its arrival. *Journal of Virology*, 92(2): e01374-17. DOI: <https://www.doi.org/10.1128/JVI.01374-17>
- Neill JD (1992). Nucleotide sequence of the capsid protein gene of two serotypes of San Moguel sea lion virus: identification of conserved and nonconserved amino acid sequences among calicivirus capsid proteins. *Virus Research*, 24: 211-222. DOI: [https://www.doi.org/10.1016/0168-1702\(92\)90008-W](https://www.doi.org/10.1016/0168-1702(92)90008-W)
- Puggioni G, Cavadini P, and Maestrale C (2013). The new French 2010 rabbit hemorrhagic disease virus Causes an RHD-like disease in the Sardinian Cape hare (*Lepus capensis mediterraneus*). *Veterinary Research*, 44: 96. DOI: <https://www.doi.org/10.1186/1297-9716-44-96>
- Salman OGA (2007). Further studies on haemorrhagic viral disease in rabbits in Egypt Ph.D. Thesis, Department of Bird and Rabbit Diseases, Faculty Veterinary Medicine Cairo University.
- Thompson JD, Higgins DG, and Gibson TJ (1994). CLUSTAL W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Research*, 22(22): 4673-4680. DOI: <https://www.doi.org/10.1093/nar/22.22.4673>
- Urakova N, Hall R, Strive T, and Frese M (2019). Restricted host specificity of rabbit hemorrhagic disease virus supported by challenge experiments in immune-compromised mice (*Mus musculus*). *Journal of Wildlife Diseases*, 55(1): 218-222. DOI: <https://www.doi.org/10.7589/2018-03-067>
- World Organization for Animal Health (OIE) (2018). Viral hemorrhagic disease of rabbits. En: *Manual of diagnostic tests and vaccines for terrestrial animals (mammals, birds, bees)*. Paris: OIE, pp. 1389-1406. Available at: https://www.oie.int/fileadmin/Home/eng/Health_standards/tahc/2018/en_chapitre_rabbit_haemorrhagic_disease.htm
- World Organization for Animal Health (OIE) (2019). Use of animal in research and education. In *terrestrial animals health code*, chapter 7.8. OIE, Paris, France. Available at: https://www.oie.int/fileadmin/Home/eng/Health_standards/tahc/2018/en_chapitre_aw_research_education.htm
- XU WY (1991). Viral hemorrhagic disease of rabbits in the people's republic of China: Epidemiology and virus characterization. *Revue Scientifique Et Technique De L'Office International Des Epizooties*, 10(2): 393-408. DOI: <https://www.doi.org/10.20506/RST.10.2.559>