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Epidemiology of Rabbit Hemorrhagic Disease Virus in Different Egyptian Governorates during 2021–2022

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ABSTRACT

Rabbit hemorrhagic disease (RHD) outbreaks have occurred in Egyptian rabbit flocks resulting insignificant mortalities and severe economic losses. During 2021–2022, 20 samples were collected from different rabbit flocks with age range of 20 days to 6 months with high mortality rates, clinical symptoms, and post-mortem lesions attributed to rabbit hemorrhagic disease. The examined rabbit flocks were in Lower Egypt governorates including Qalubia, Sharkia, Kafr ElSheikh, Dakhlia, Gharbia, and Behira, and in Upper Egypt governorates including Giza, Menia, Assuit, and Sohag. The present investigation aimed at molecular identification of the current rabbit hemorrhagic disease virus (RHDV) strains detected in distinct rabbit populations in various Egyptian governorates. Hemagglutination (HA) test and molecular characterization using a one-step reverse transcriptase-polymerase chain reaction (RT-PCR) targeting the partial VP60 of RHDV were utilized for identifying these agents. Sequencing and phylogenetic analysis were successfully conducted for genotyping RHDV strains. 17/20 (about 85%) farms were RHDV-positive, which was confirmed using HA test and RT-PCR. Two genotypes were identified through partial sequencing of theVP60 gene, six strains were clustered to RHDV2/b, and the other three strains were clustered to the RHDVa strain. According to the current study, RHDV2 has become the predominant strain threatening the rabbit populations in Lower Egypt governorates. On the contrary, the RHDVa strain continues to be a hazard to the rabbit flocks in Upper Egypt governorates including Giza, Menia, and Assuit.

Key words: Egypt, RHDV, HA test, RT-PCR, Sequencing, VP60.

INTRODUCTION

Rabbit hemorrhagic disease (RHD), a highly lethal contagious disease affecting domestic and wild rabbits, poses a threat to the rabbit industry in Egypt and worldwide due to its high fatality rates (Dalton et al. 2015; Magouz et al. 2019; Abd El-Moaty et al. 2020).The etiological agent RHDV is an icosahedral, non-enveloped, positive-sense, single-stranded RNA virus within the genus *Lagovirus* and family Caliciviridae (Abrantes et al.2012).

RHDV pathogenic isolates are clustered into three categories: the "classic RHDV", including isolated genogroups G1–G5; the variant RHDVa/G6; and the novel type RHDV2/RHDVb (OIE 2021). Initial clinical cases of RHD were recorded in China in 1984 (Xu and Chen 1989) and subsequently spread globally (Kesy et al. 1996).

In 1996, in Italy and Germany, a new variant strain RHDVa was detected in different breeds of rabbits (Capucci et al. 1998). In 2010, in France, the emergence of new RHDV strain called RHDV2 was detected in rabbits of various ages. RHDV2 spread rapidly throughout Europe, Australia and Sweden (Le Gall-Reculé et al. 2011; Mahar et al. 2018; Rouco et al. 2018).

In Egypt, the initial detection of the disease was in El-Sharkia governorate in the spring of 1991 (Ghanem and Ismail 1992). Then, the virus was detected in Qalubia governorate (Sharawi 1992) and in other Egyptian governorates (Ghanem and Ismail 1992). Since 2007, RHDVa variant strains have been registered in Egypt (Ewees 2007; El-Sissi and Gafer 2008). Until now, these variant strains have caused several outbreaks, recorded in New Valley and Assuit in Upper Egypt (Abodalal et al. 2021).

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The new *Lagovirus* RHDV2 was identified in a number of Lower Egypt governorates in 2018 and 2019 (Abodalal and Tahoon 2020; Erfan and Shalaby 2020; Hemida et al. 2020).

The incubation period of RHDV ranges from16 to 48 hours, with mortalities occurring within 2 to 3 days after infection. The severity of clinical symptoms depends on the animals breed, age, immunity, geographical distribution, infecting virus dose, and infection methods. In the pre-acute stage, healthy animals abruptly die within 12 to 36 hours after the onset of the disease without showing any preceding clinical signs (Belz 2004; Calvete et al. 2018).

Fever, depression, frothy bloody nasal discharge, epistaxis, and pregnant does showed vulvar hemorrhages and respiratory manifestations including cough and dyspnea, in addition to eventually neurological signs (ataxia, convulsion, opisthotonos, and paralysis) which appeared at the acute stage of the disease (Trzeciak-Ryczek et al. 2015). Meanwhile, sub-acute and chronic stages are denoted by severe jaundice, emaciation, constipation, or diarrhea and distension of the abdomen and then the death of the animal in a few weeks (Capucci et al. 1991).

According to Le Gall-Reculé et al. (2013), the RHDVa variant strains and newly emerging RHDV2 strains are both circulating in rabbit populations with similar symptoms and lesions to those of classic RHDV. However, the mortality in RHDVa infected rabbits may be relatively lower. RHDV2 is characterized by a fluctuating mortality rate in experimentally infected rabbits ranging from 5 to 70% with an average of 20%. Death can occur as early as 15 days of age in adult and lactating rabbits (OIE 2018).

Strains of RHDV are characterized by a high genetic mutation rate but of one serotype (Gould et al. 1997). Both RHDV and RHDV2 genomic structures are identical and contain two open reading frames (ORFs). ORF1 encodes nonstructural proteins, including the RNA-dependent RNA polymerase and the major capsid protein (VP60). On the contrary, the second ORF encodes a minor structural protein known as VP10 (Dalton et al. 2015; Meyers et al. 2000). Furthermore, RHDV is RNA positive-sense with additional structural proteins (sub-genomic RNA) of around 2.2 kb, essential for later phases of infection (Abrantes et al. 2012). VP60 is the immunogenic protein and the primary viral structure of RHDV (Mikschofsky 2009; OIE 2019), comprising three parts: the N-terminal arm, S, and a short hinge, and P split into two portions, P1 and P2. The virus domain P is required for host cells attachment, whereas the P2 sub-domain is necessary for genetic variation (Wang et al. 2013).

In addition, VP60 has been chosen as a target for reverse-transcription PCR (RT-PCR) which can be utilized for detecting viruses that are difficult to identify using conventional technique (Le Gall-Reculé 2017; Kwit and Rzeżutka 2019). The genetic variation among RHD viruses is primarily based on the sequence of VP60 gene (Le Gall-Reculé et al. 2003; Forrester et al. 2006; McIntosh et al. 2007; Forrester et al. 2008; Wang et al. 2013).

Based on the phylogenetic analysis, the RHDV strains can be categorized into three types: classical RHDV with genogroups G1–G5, RHDVa/G6 variant strain, and the novel type RHDV2/RHDVb (Le Gall-Reculé et al. 2013; Qi et al. 2019).

The current investigation aimed to identify RHDV strains circulating in distinct rabbit flocks in Lower and Upper Egypt governorates via molecular identification in order to detect the relationship of any novel strain to the routinely utilized isolates for the essential vaccination programs.

MATERIALS AND METHODS

Ethics Statement

The Institutional Animals Care and Use Committee, Research Ethics Board, Faculty of Veterinary Medicine, Benha University (No. BUFVTM 331022), approved the study protocols, following animal welfare guidelines.

Samples Collection

Liver samples were collected from RHD-suspected freshly dead rabbits with age range of 20–35 days for suckling rabbits, 35–55 days for weaning rabbits, 55 days up to 4 months for growing rabbits, and more than 4 months for adult rabbits. About 3:4 liver tissues were collected from each farm and pooled with each other representing one sample. Aseptically collected samples were packed in sterile jars and sent to the laboratory for subsequent diagnosis. As mentioned in Table 1 and Fig.1, the samples were taken from several flocks in ten Egyptian governorates. The examined rabbits suffered from depression, off-food, blood-stained frothy nasal discharge, and sudden death, while during post-mortem (PM) examination freshly dead rabbits showed lobular necrosis of the liver, congested hemorrhagic spleen, and hemorrhagic kidneys, as well as hemorrhagic tracheitis.

Sample Preparation

Liver tissue is homogenized mechanically in phosphate-buffered saline (PBS) solution of 5–20% (w/v) and pH 7.2–7.4. Then, freezing-thawing was repeated three times followed by centrifugation at 5000g for 15min (OIE 2021).

Hemagglutination (HA) Test

According to OIE (2021), a HA test was conducted. In brief, HA was carried out through the use of serial two-fold dilutions of prepared liver tissue in 50µL PBS, pH 7.2, with positive and negative controls included. 50µL of 1% washed human type "O" red blood cells was applied to each well and was then incubated at 4°C for 1h. The HA titer was evaluated with the reciprocal of the highest dilution capable of causing RBCs hemagglutination.

Reverse Transcriptase-Polymerase Chain Reaction RNA Extraction

Viral RNA extraction was carried out using the QIAamp viral RNA Mini kit (QIAGEN, Valencia, California, USA) (Cat. no. 52906), according to the manufacturer's instructions.

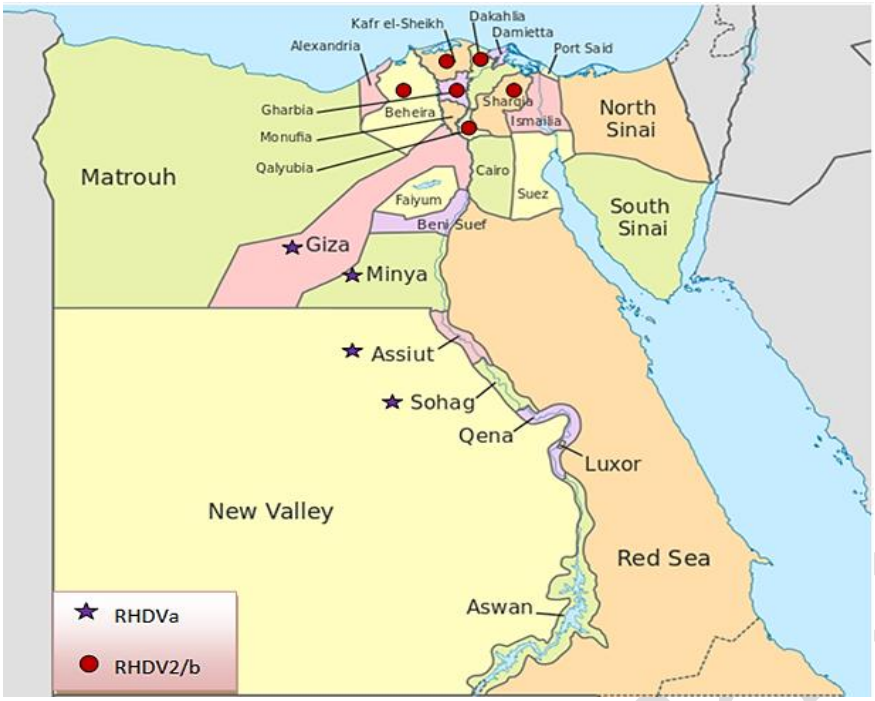

Fig. 1: Egyptian epidemiological map showed the geographical distribution of collected samples for identification of RHDVduring 2021–2022. RHDV1 virus appeared as violet stars in Upper Egypt (Except Sohag) while RHDV2 appeared as red dots in Lower Egypt.

Table 1: The flock history of suspected RHDVa cases

Sample number	Date	Governorate	Vaccination status	Farm capacity Dams	Mortality%			
					Suckling[1]	Weaning[2]	Growing[3]	Adult[4]
1	28/8/2021	Qalubia	No	40	40	30	50	50
2	5/9/2021	Kafr El Sheikh	No	70	70	80	60	50
3	25/9/2021	Sharkia	Vaccinated*	50	80	70	60	30
4	11/10/2021	Kafr El Sheikh	No	50	30	20	42	20
5	20/10/2021	Qalubia	No	50	70	70	40	30
6	1/11/2021	Kafr El Sheikh	No	70	60	50	50	40
7	17/11/2021	Sharkia	No	40	80	70	20	40
8	28/12/2021	Kafr El Sheikh	Vaccinated*	60	50	40	20	20
9	10/12/2021	Qalubia	Vaccinated*	20	100	80	40	40
10	3/1/2022	Qalubia	No	10	90	80	40	50
11	10/1/2022	Qalubia	No	80	70	80	60	40
12	22/1/2022	Dakhlia	Vaccinated*	50	50	40	30	40
13	10/2/2022	Gharbia	No	70	60	60	50	40
14	21/2/2022	Kafr El Sheikh	No	40	80	70	80	80
15	27/1/2022	Behira	No	80	80	70	70	50
16	1/3/2022	Qalubia	Vaccinated*	30	60	50	30	50
17	5/8/2022	Assuit	No	50	30	20	50	80
18	10/8/2022	Giza	Vaccinated**	60	15	20	40	40
19	25/8/2022	Menia	No	70	30	25	80	90
20	1/9/2022	Sohag	Vaccinated*	70	40	50	50	50

1Suckling rabbits aged 20–35 days. 2Weaning rabbits aged 35–55 days. 3Growing rabbits aged 55 days up to 4 months.4Adult rabbits aged more than 4 months. *Vaccinated with RHD Vaccine. **Vaccinated with imported RHDVa vaccine.

PCR Amplification

The designed primer was utilized for amplifying VP60 targeting 624bp (P33: 5′-CCTGGAGGGTTTTCTACGTG -3′ and P34: 5′-AGACGACAGACGCGAACAT -3′). The reactions were applied through a one-step reverse transcriptase-polymerase chain reaction (RT-PCR). Amplification parameters were reverse transcription at 50°C for 30min, followed by a primary denaturation at 95°C for 15min, 40 cycles of 95°C for 30s, 52°C for 45s, and 72°C for 1min. Finally, extension step was carried out at 72°C for 10min.

Sequencing of VP60 Gene and Phylogenetic Analyses

Purification of PCR products was conducted according to the manufacturer's kit (QIA quick PCR product extraction kit). Partial sequencing of the VP60 gene in two directions was carried out through the use of a Big Dye Terminator V3.1 cycle sequencing kit (Foster City, USA) (Cat. no. 433693VP60). Sequences were acquired using a 3130 genetic analyzer. MEGA version 7 (www.megasoftware.net) was utilized for assembling the VP60 gene nucleotide sequences, which were then compared with the other representative sequences available at GenBank. The phylogenetic tree was

constructed using the maximum likelihood tree method with moderate strength and 1000 bootstrap replicates. The identity percent of nucleotide and amino acid was evaluated through the use of DNA star software (DNA Star, Madison, WI).

RESULTS

Haemagglutination (HA) Assay

HA was performed on the liver homogenates against human-type "O" red blood cells. The result revealed that 17 out of 20 samples were positive, with titer varying from 2^9 to 2^{12}.

Reverse Transcriptase-Polymerase Chain Reaction

Through a one-step RT-PCR, the presence of the RHDV virus was verified in all samples, HA positive or negative. It was identified that 17 (85%) out of 20 samples were RT-PCR positive for the RHDV virus, with the presence of an amplified band at 624bp as illustrated in Fig. 2.

Sequencing of VP60 Gene and Phylogenetic Analyses

Nine molecular identified strains were selected for sequencing and phylogenetic analysis from 17 positive RHVD isolates, according to the governorate and age and from either vaccinated or unvaccinated farms. Nucleotide sequencing and alignment were made of partial VP60 gene (624 bp) and tested for their amino acid identity % in comparison with reference strains sequences obtained from GenBank via their accession number. Sequencing of the VP60 amplicons revealed that two different RHDV strains were circulating in different Egyptian governorates. Six isolates were closely related to RHDVb/2, and the other three isolates were clustered to RHDVa in comparison with RHDVa strains available from GenBank (Fig. 3).

The nucleotide sequence and identities of amino acid of six isolates (RHDV2) were 98–98.8% in comparison with the other RHDV2 available strains, while the identity of the other three isolates (RHDVa) were 98.4–99.4% in comparison with the other available RHDVa strains on GenBank. As demonstrated in Fig. 4 and 5, our RHDV2 isolates had 97.4–100% amino acid identity, while RHDVa isolates displayed 99–100% amino acid identity, these nine identified strains were assigned on GenBank and had accession numbers as presented in Table 2.

DISCUSSION

RHD is one of the most severe infectious diseases causing devastating outbreaks in the rabbit population worldwide. Nowadays, RHDV2 has gradually become the predominant strain in rabbit flocks in addition to the classic strain still threatening the rabbit flocks resulting in severe economic losses to the rabbit sector. The disease becomes endemic among rabbits of different ages despite using various vaccination strategies (Kwit and Rzeżutka 2019; Hemida et al. 2020).

Our current study was conceded to characterize the RHDV strains responsible for massive outbreaks in rabbit farms in 10 Egyptian governorates during 2021–2022, the investigated rabbit flocks suffered from sudden death and blood-stained frothy nasal discharge, in addition to lobular liver necrosis and generalized congestion and hemorrhages. These findings confirmed the suspicions of RHDV infection as previously mentioned by numerous authors (Alonso et al. 1998; Marques et al. 2010; El-Samadony et al. 2021).

In the present study, 17 out of 20 cases were RHDV-positive which was confirmed using the HA test and PCR. Firstly, the prepared samples were tested by HA at 4°C using human RBCs type O. About 17 samples were positive, with titer varying from 2^9 to 2^{12}. These results coincided with the study of Abido et al. (2020), who detected that 10 out of 11 samples were positive through HA, with titer ranging from 8 to 13 log2. With the presence of non-hemagglutinating strains of RHDV, the sensitivity and specificity of the HA test appear to be inadequate (Ewees 2007; El-Sissi and Gafer 2008). Thus, the use of RT-PCR was a rapid and more sensitive method for detecting RHDV. One-step RT-PCR targeting the VP60 was utilized for the detection of RHDV in suspected cases. The result demonstrated that 17 out of 20 samples were RHDV-positive.

Nucleotide sequence and phylogenetic studies, representing the primary molecular determinant of RHDV genotype, were performed on the selected nine isolates, which revealed that six isolates were clustered with

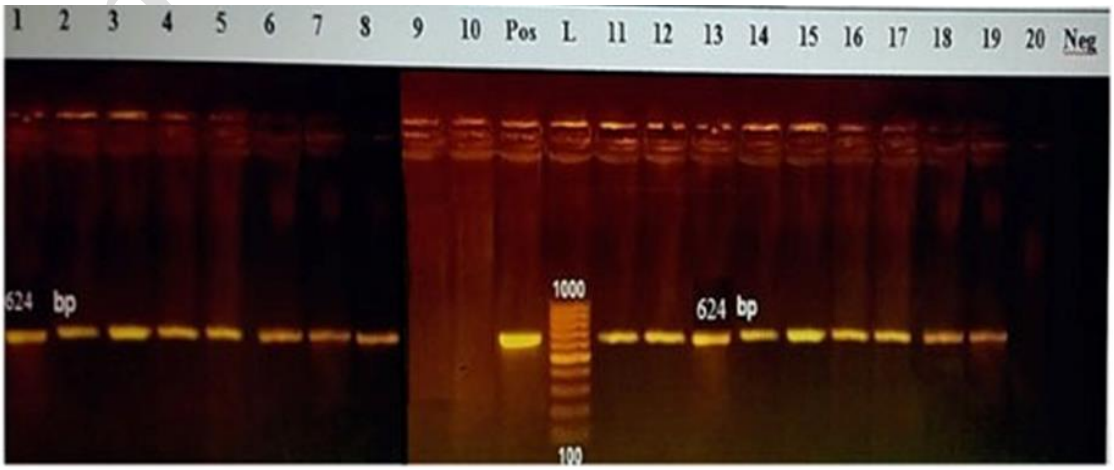

Fig. 2: Gel electrophoresis of 624bp fragment from VP60 gene of RHDV isolates.

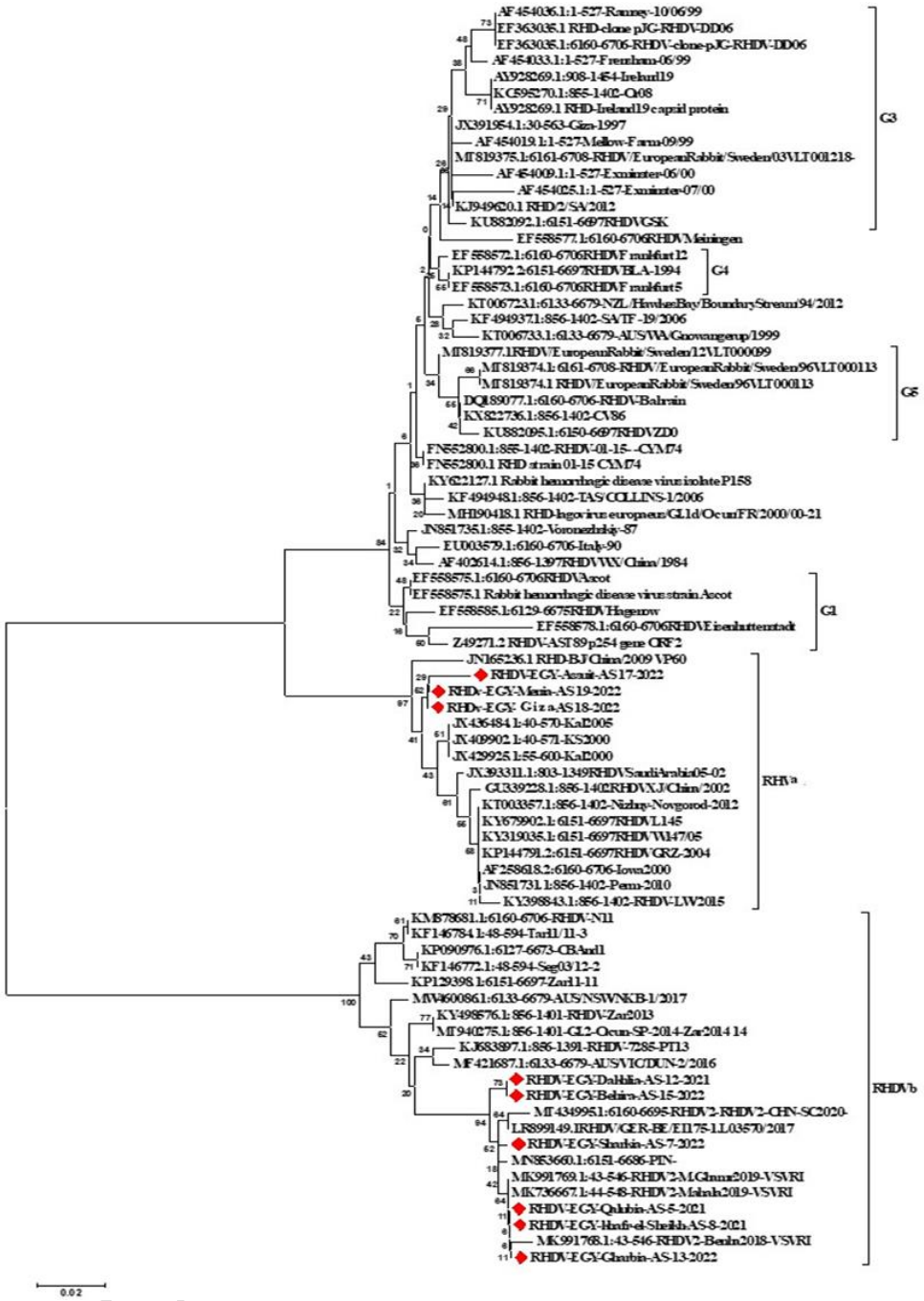

Fig.3: Phylogenetic tree of the 9 RHDV isolates based on partial nucleotides sequence of the VP60 gene.

AMINO ACID similarity

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	
1		96.0	96.0	93.8	91.1	90.5	77.2	77.4	78.0	78.0	77.0	77.6	77.4	77.8	77.8	78.4	77.8	90.5	91.5	91.5	1
2	4.1		96.4	96.0	90.1	89.1	76.6	76.8	77.4	77.4	76.4	77.0	77.2	76.8	77.2	77.8	77.2	89.5	90.5	90.5	2
3	4.1	3.7		94.6	90.3	88.9	78.8	79.0	79.2	78.8	77.8	78.8	79.0	78.6	79.0	79.6	79.0	89.7	90.7	90.7	3
4	6.5	4.1	5.6		87.9	86.5	77.0	77.2	77.2	77.4	76.8	77.0	77.2	76.8	76.8	77.8	76.8	87.3	88.3	88.3	4
5	9.8	10.9	10.7	13.6		95.2	76.2	76.2	76.4	76.4	76.8	76.0	76.2	75.8	77.0	76.4	77.0	98.4	99.4	99.4	5
6	10.4	12.1	12.3	15.4	5.0		77.2	77.2	76.8	76.8	77.8	76.6	76.6	76.4	77.4	76.8	77.4	94.4	95.4	95.4	6
7	28.4	29.4	25.8	28.6	30.2	28.4		98.6	95.6	95.6	96.4	95.8	95.8	95.4	95.2	95.2	95.2	76.2	76.8	76.8	7
8	28.1	29.1	25.5	28.2	30.1	28.3	1.4		96.2	96.6	96.6	96.4	96.4	96.0	95.8	95.8	95.8	76.2	76.8	76.8	8
9	27.1	28.0	25.2	28.2	29.7	29.0	4.5	3.9		99.2	96.2	98.6	98.6	98.6	98.0	98.8	98.0	76.4	77.0	77.0	9
10	27.1	28.1	25.8	27.9	29.8	29.0	4.5	3.5	0.8		96.6	98.6	98.6	98.6	98.0	98.8	98.0	76.4	77.0	77.0	10
11	28.7	29.7	27.4	28.9	29.1	27.4	3.7	3.5	3.9	3.5		96.6	96.2	96.4	96.0	95.8	96.0	76.8	77.4	77.4	11
12	27.7	28.7	25.8	28.6	30.5	29.3	4.3	3.7	1.4	1.4	3.5		98.4	99.6	97.8	98.6	97.8	76.0	76.6	76.6	12
13	28.0	28.3	25.5	28.2	30.1	29.3	4.3	3.7	1.4	1.4	3.9	1.6		98.0	99.0	98.2	99.0	76.2	76.8	76.8	13
14	27.4	29.1	26.2	28.9	30.8	29.7	4.8	4.1	1.4	1.4	3.7	0.4	2.0		97.4	98.6	97.4	75.8	76.4	76.4	14
15	27.4	28.3	25.5	28.9	28.7	28.0	5.0	4.3	2.0	2.0	4.1	2.2	1.0	2.6		97.6	100.0	77.0	77.6	77.6	15
16	26.5	27.4	24.6	27.3	29.8	29.0	5.0	4.3	1.2	1.2	4.3	1.4	1.8	1.4	2.4		97.6	76.4	77.0	77.0	16
17	27.4	28.3	25.5	28.9	28.7	28.0	5.0	4.3	2.0	2.0	4.1	2.2	1.0	2.6	0.0	2.4		77.0	77.6	77.6	17
18	10.4	11.6	11.3	14.3	1.6	5.8	30.0	29.9	29.5	29.6	28.9	30.2	29.8	30.6	28.5	29.6	28.5		99.0	99.0	18
19	9.3	10.5	10.2	13.1	0.6	4.8	29.3	29.2	28.8	28.8	28.2	29.5	29.1	29.8	27.8	28.8	27.8	1.0		100.0	19
20	9.3	10.5	10.2	13.1	0.6	4.8	29.3	29.2	28.8	28.8	28.2	29.5	29.1	29.8	27.8	28.8	27.8	1.0	0.0		20
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	

1 EU003579.1:Italy-90-G1
2 MH190418.1RHDV/Europaeus-GI.1d-FR-2000
3 EF558573.1RHDV-Frankfurt5-G4
4 DQ189077.1RHDV-Bahrain-G5
5 RHDV-OZA-2006-RHDV-o
6 KY398843.1RHDVALW-2015-G2
7 KM878681.1N11-RHDVb
8 MT940285GI.2Ocun-SP-2014-Zar2014
9 MK991768.1Benin2018VSVRI
10 RHDV-Mahdia2019-VSVRI
11 MW460084.1AUSNSW-MURR-2019
12 RHDV-EGY-Qalybia-AS-5-2021
13 RHDV-EGY-Sharkia-AS-7-2022
14 RHDV-EGY-KhafrelSheikh-AS8-2021
15 RHDV-EGY-Qalubia-AS-12-2021
16 RHDV-EGY-Gharbia-AS-13-2022
17 RHDV-EGY-Behira-AS-15-2022
18 RHDV-EGY-Assuit-AS-17-2022
19 RHDv-EGY-Giza-AS-18-2022
20 RHDv-EGY-Menia-AS-19-2022

Fig.4: Amino acid identity of RHDV isolates with RHDV strains and RHDV vaccines.

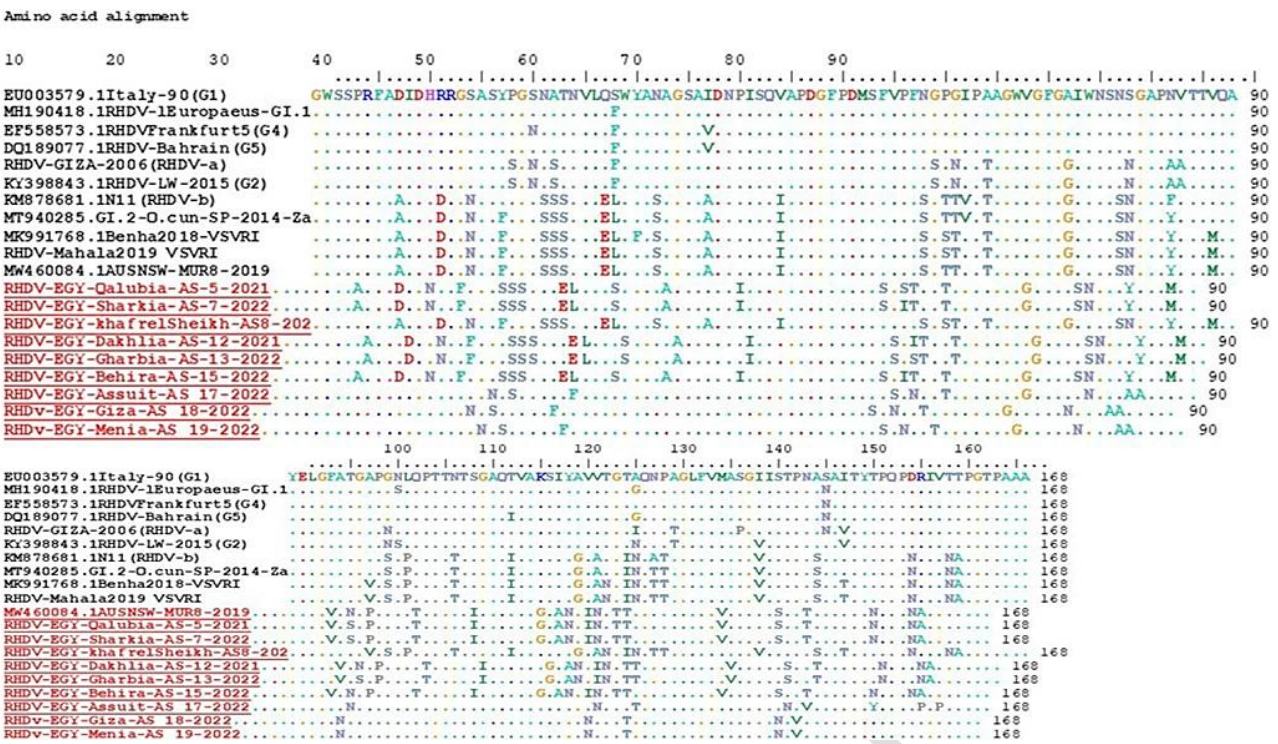

Fig. 5: Deduced amino acid sequences of VP60 protein of the isolated RHDV strains in comparison with other RHDV isolates selected from GenBank.

Table 2: Molecular identified strains with their accession number.

Serial number	Abbreviation	Genotype	GenBank Accession Number
1	RHDV-EGY-Qalubia-AS 5-2021	RHDV2	ON920552
2	RHDV-EGY-Sharkia-AS 7-2022	RHDV2	ON920553
3	RHDV-EGY-Kafr El Sheikh-AS 8-2021	RHDV2	ON920554
4	RHDV-EGY-Dakhlia-AS 12-2021	RHDV2	ON920555
5	RHDV-EGY-Gharbia-AS 13-2022	RHDV2	ON920556
6	RHDV-EGY-Behira-AS 15-2022	RHDV2	ON920557
7	RHDV-EGY-Assuit-AS 17-2022	RHDVa	OP554373
8	RHDV-EGY-Giza-AS 18-2022	RHDVa	OP554374
9	RHDV-EGY-Menia-AS 19-2022	RHDVa	OP554375

Egyptian RHDVb/2 strains. In comparison, the other three isolates were closely related to RHDVa strains, as demonstrated in Fig. 3 and 4. The result indicated that either RHDV2 or RHDVa was still circulated in rabbit populations of different ages and breeds in addition to vaccinated or nonvaccinated flocks. This result was consistent with the findings of Le Gall-Reculé et al. (2011) and Peacock et al. (2017), who reported that the RHDV could cause the disease in vaccinated and young rabbits, in addition to the findings of El-Samadony et al. (2021), who reported that the virus could cause RHD in vaccinated or nonvaccinated rabbits. Furthermore, this result revealed that the new *Lagovirus* RHDV2 became the predominant strain in Lower Egypt governorates, whereas RHDVa still circulated in rabbit flocks, especially in Upper Egypt governorates.

This result agreed with the study of Erfan and Shalaby (2020), who successfully identified the classical RHDV strain in Upper Egypt, while RHDV2 was confirmed mainly in Lower Egypt governorates.

Conclusion

The current study reports the presence of RHDVa and RHDV2 circulating among rabbit flocks in Egypt, with the breakage of vaccination programs causing an economic loss in the rabbit industry. Therefore, there is an urgent need to utilize a bivalent vaccine that helps in controlling the disease. In addition, continuous monitoring of RHDV molecular epidemiology with complete genome sequences of the virus is recommended for planning new effective vaccination strategies.

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Author's Contributions

All authors designed the study, Asmaa Ibrahim Mohamed Desouky and Samah El Sayed Ali Abodalal performed the practical procedures, analyzed and interpreted the data, and wrote the manuscript. This manuscript content was authored, reviewed, and approved by Asmaa Ibrahim Mohamed Desouky, Amal HassanTawfik Abdelnaser, Magda Mohamed Ali Moustafa, Ibrahim Mohamed Elboraay, and Samah El Sayed Ali Abodalal for publication.

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