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Original article

PREVALENCE OF RABBIT HAEMORRHAGIC DISEASE VIRUS 2 IN DELTA AND UPPER EGYPT

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Summary

Elsayed, E. A., S. E. A. Abodalal, A. Y. Tahoon, M. Fawzy & M. S. El-Shahidy, 2023. Prevalence of rabbit haemorrhagic disease virus 2 in Delta and Upper Egypt. *Bulg. J. Vet. Med.* (online first).

Rabbit viral haemorrhagic disease (RVHD) is a fatal threat to rabbits causing long-term problems and significant economic losses. In the current study, RVHD was identified and characterised in naturally infected rabbits in order to assess the genetic diversity of RHDV circulating in different Egyptian provinces from January 2019 to January 2022. Nineteen suspected samples were collected from outbreaks that occurred in nine provinces during 2019–2022. Ten liver samples out of nineteen were positive in the slide and plate haemagglutination (HA) test. HA titres ranged from 5 $\log_2$ to 12 $\log_2$. RHDV-positive liver homogenates were confirmed with RT-PCR and histopathology. Further characterisation of the selected four viral strains was performed by nucleotide sequencing of *VP60* gene. Based on nucleotide sequence analysis, three isolates were identified as RHDV2 strains, while one isolate was assigned as RHDV1 strain.

Key words: Delta, phylogenetic analysis, RHDV2, VP60

INTRODUCTION

Rabbit viral haemorrhagic disease (RVHD) is a highly contagious and acute fatal hepatitis viral disease affecting wild and domestic European rabbits. RVHD has a significant economic impact due to high mortality as well as great losses in meat and fur production in Egypt (Dalton *et al*., 2015). RVHD is caused by *Calicivirus* (RHDV) which belongs to genus *Lagovirus,* family *Calciviridae.* It is a spherical, non-enveloped, small-sized RNA virus with a major capsid protein (VP60) and positive-sense, single-stranded RNA genome (Abrantes *et al*., 2012). RHDV isolates have been classified into three subtypes: classic RHDV

(G1–G5), RHDVa (G6), and RHDVb (G1.2) (Dalton *et al*., 2012; Le Gall-Reculé *et al*., 2013). Lately, they have been categorised into GI which includes GI.1 and G1.2. GI.1 has different forms of classic strains and has been reclassified into GI.1a (G6/RHDVa), GI.1b (G1), GI.1c (G2), and GI.1d (G3–G5) (Le Pendu *et al*., 2017).

Death occurs in lactating rabbits from 15 days of age onwards, and the course of the disease is usually longer (3–5 days), with a higher proportion of rabbits showing subacute or chronic disease (Puggioni *et al*., 2013). The first record of RHDV in Egypt was in Sharkia governorate in 1991, where the virus was associated with 90% of the observed mortality (Ghanem & Ismail, 1992). Later, RHDV was recorded in Qalubia governorate (Sharawi, 1992). In Upper Egypt, RHDV was detected in Assiut governorate in 1992 (Salem & El-Ballal, 1992). Later, subsequent disease outbreaks have been recorded in different Egyptian governorates (El-Zanaty, 1994). Until 2010, all isolated RHDV belonged to one of the six identified genotypes (G1–G6), where G6 is an antigenic subtype (RHDVa). In 2010, a new virus RHDV2 was identified in France, phylogenetically and antigenically distinct from RHDV. The RHDV2 variant (GI.2/RHDV2/b) emerged in France in 2010 (Le Gall-Reculé *et al*., 2013) and was responsible for massive declines in the European rabbit populations (Delibes-Mateos *et al*., 2008). The new antigenic variant of RHDV was introduced to Egypt and spread to most of the Egyptian provinces (Erfan & Shalaby, 2020; Hemida *et al*., 2020).

Both viruses causing RHD are extremely contagious. Transmission occurs by direct contact with infected animals, carcasses, body fluids (urine, faeces, and respiratory secretions), and hair. Surviving rabbits may be contagious for up to 2 months (Abrantes *et al*., 2012). Thus, the current study aims to detect RHDV1 and RHDV2 in some provinces of Lower and Upper Egypt as well as to determine the genetic relationship between the Egyptian isolates for better vaccination strategies designing.

MATERIALS AND METHODS

Ethical approval

All procedures conducted in the current work were carried out according to the guidelines and requirements of ethical approaches in dealing with experimental animals in research and according to the ten principles of the Declaration of Helsinki (OIE, 2018). All examined tissues were disposed according to the biosafety procedures under the Egyptian committees of animal welfare supervision.

Rabbit flocks, clinical and post-mortem examination

Suspected RVHD outbreaks were observed in 19 rabbit flocks in 9 Egyptian governorates: 4 flocks in Kafr Elsheikh province, 8 flocks in Gharbia province, and one flock in each of Damietta, Menofia, Dakahlia, Qalubia, Behira, Assiut, and Sohag provinces. The freshly dead rabbits during the suspected RHDV were collected from rabbit flocks exhibiting symptoms and lesions of RHDV during 2019–2022. The investigated rabbitries were clinically examined during the outbreaks and were subjected to post mortem examination along with recording of the observed macroscopic pathological findings.

Some of the investigated rabbitries were previously vaccinated with either local or imported vaccines and others were

not vaccinated against RHDV1. The investigation data are illustrated in Table 1.

Samples preparation

Liver tissues were aseptically collected from freshly dead rabbits. Liver extract was prepared after homogenisation of 10% (w/v) liver tissue samples in PBS according to OIE (2021).

Haemagglutination (HA) test

Washed human type "O" erythrocytes, were suspended in sterile saline at 0.75% and 10% for HA micro-technique and rapid slide HA tests respectively. Two-fold dilutions of homogenised liver tissue PBS suspension (10% w/v) were incubated with an equal volume of washed human red blood cells (RBCs) type "O" (0.75% concentration) in a V-shaped-bottom micro-titre plate at 4 °C according to OIE (2021).

Isolation and identification of RHDV

Isolation and identification of RHDV were performed in susceptible rabbits as reported by OIE (2021). Liver extracts from the freshly dead rabbits were inoculated (1 mL/rabbit I/M) into 5 susceptible cross-breed rabbits (aged 1 month and seronegative for RHDV HI antibodies). Another 5 rabbits were inoculated with 1 mL sterile saline solution and kept as negative controls.

RNA extraction

Total RNA was extracted from liver tissues using a DNase nucleic acid extraction reagent, QIAamp viral RNA Mini Extraction Kit, Spin Column (QIAGEN, Valencia, California., USA) (Cat. No 52906), according to the manufacturer's instructions.

Reverse transcription/polymerase chain reaction (RT/PCR)

RHDV RNA was detected in the examined samples. The designed primer was utilised for amplifying *VP60* targeting a 600 bp fragment:

- RHDV- F CCTGGAGGGTTTTCTAC GTG
- RHDV-R AGACGACAGACGCGAA CAT

As demonstrated by Meyers *et al*. (2000), the samples were applied through a one-step reverse transcriptase-polymerase chain reaction (RT-PCR). Amplification parameters included reverse transcription at 50 °C for 30 min, followed by initial denaturation at 95 °C for 3 min, 35 cycles of 95 °C for 30 s, 52 °C for 30 s, and 72 °C for 1 min. Finally, an extension step was carried out at 72 °C for 5 minutes. Electrophoresis of RT-PCR products was performed using 2% agarose gel.

VP60 gene sequencing and phylogenetic analyses

Purification of PCR products was performed according to the manufacturer's kit (QIAquick PCR Product Extraction Kit, QIAGEN, Germany), as described in the manufacturer's protocol.

Partial sequencing of the *VP60* gene in two directions was carried out using a Bigdye Terminator Cycle Sequencing Kit (Foster City, USA). *VP60* sequences were acquired through the use of a 3130x1 genetic analyzer. The generated RHDV-VP60 gene nucleotide sequences were assembled using Geneious© Software (http://www.geneious.com) and aligned with representative sequences from GenBank using MAFFT. The identity percentage was calculated, and phylogenetic trees were constructed using the UPGMA method (Sneath & Sokal, 1973) and em-

ploying the Jukes-Cantor model (Jukes & Cantor, 1969).

RESULTS

A total of 19 rabbit flocks raised in 9 Egyptian governorates were examined for mortalities, clinical signs, and post-mortem lesions. The mortality rate in the investigated rabbitries ranged from 30 to 90% in suckling rabbits aged 17–35 days. In the adult rabbits aged more than 4 months, the mortality rate ranged from 20% to 50%, whereas in the growing rabbits aged 55 days up to 4 months, the morality varied from no mortality (0%) to 70% (Table 1). Variable clinical signs were observed on the affected rabbits at the investigated rabbitries (Kafr Elsheikh, Gharbia, Damietta, Assiut, and Sohag) as follows: pyrexia with increased respiratory rates as well as cyanosis of lips and nostrils. Suckling and weaning rabbits exhibited haemorrhagic nasal discharge and convulsions and other neurological signs such as ataxia and paddling with legs seen just before death.

Occasionally, the dead rabbits were found in opisthotonus position (spasm of the muscles causing backward arching of the head, neck, and spine). Sometimes the anal sphincter appeared loosened and with mucoid faecal discharges. The most consistent post mortem lesion was the haemorrhage in almost all organs accompanied by poor blood coagulation. The most severely affected organ was the liver (brownish and friable). Meanwhile, in weaning rabbits, the liver sometimes appeared pale with icteric discoloration, and the trachea was often full with a foamy bloody exudate. The lungs showed congestion and oedema with multifocal punctate haemorrhages of variable size accompanied by subpleural haemorrhages. The spleen was swollen, severely congested, and enlarged 2–3 times with rounded edge. The kidneys were dark brown, hyperaemic and enlarged. The urinary bladder was found full with turbid urine.

The haemagglutination (HA) tests showed that 10 out of 19 liver samples (3 Kafr Elsheikh, 4 Gharbia, 1 Damietta, 1 Assiut, and 1 Sohag) were positive with slide and plate HA test. HA titres ranged from 5 $\log_2$ to 12 $\log_2$. Positive liver homogenates were confirmed as RHDV with RT-PCR. Ten samples proved to be positive for RHDV by slide and plate HA test were selected for amplifying the *VP60* gene with RT-PCR and gel electrophoresis. The predicted bands of RHDV were observed at 600 bp in the gel. The intensity of RT-PCR bands in the gel was utilised as symbols for selecting 4 samples for sequencing of *VP60* gene. The bands were cut, purified, and sequenced. Two sequences from Kafr Elsheikh isolates (2019 and 2020), one Damietta isolate (2020), and one Sohag isolate (2022) were utilised for phylogeny. The partial *VP60* (C to E regions) sequences of 4 isolates were submitted to Gen Bank with accession numbers as presented in Table 2.

The phylogenetic tree was constructed by the neighbour-joining method for nucleotide sequence of RHDV for the highly variable region of *VP60* gene (Fig. 1), representing 12 classical RHDV strains, 3 variant RHDVa strains, and 11 RHVD2 strains as out-group. Four isolates were analysed. The RHDV Kafr Elsheikh (2019 and 2020) and Damietta (2020) isolates were grouped together in a separate subclade and closely related to RHDV2 (GI.2) with minor variability. Meanwhile, the Sohag (2022) isolate was grouped with RHDV var 1.

Table 1. Number, localities, and mortality rates of the examined flock

Flock No./date	Governorate	Deaths / Number				Total deaths / Total number	Vaccinal status	Breed
		Suckling	Weaning	Growing	Adult			
1 (7/2019)	Kafr Elsheikh	16/30	5/10	23/50	39/80	83/170	Vaccinated	Foreign
2 (9/2019)	Kafr Elsheikh	45/50	66/80	40/75	19/40	170/245	Non	Foreign
3 (6/2020)	Kafr Elsheikh	41/65	20/30	61/90	14/20	136/205	Non	Foreign
4 (12/2020)	Kafr Elsheikh	22/50	23/60	30/70	5/10	80/190	Vaccinated	Foreign
5 (9/2019)	Gharbia	27/80	25/90	27/90	11/30	90/290	Non	Native
6 (11/2020)	Gharbia	9/20	5/10	30/60	46/90	90/180	Non	Native
7 (12/2020)	Gharbia	20/50	18/40	34/80	8/20	80/190	Non	Foreign
8 (6/2021)	Gharbia	26/60	31/80	29/70	14/30	100/240	Non	Foreign
9 (6/2021)	Gharbia	14/50	12/40	15/50	4/10	45/150	Vaccinated	Foreign
10 (7/2021)	Gharbia	28/60	13/30	25/60	4/10	70/160	Non	Foreign
11 (7/2021)	Damietta	25/40	27/50	13/40	4/10	69/140	Non	Foreign
12 (7/2021)	Gharbia	19/50	16/40	24/60	12/30	71/180	Non	Foreign
13 (8/2020)	Gharbia	35/90	12/85	33/100	9/25	89/245	Non	Foreign
14 (11/2020)	Menofia	26/80	32/90	29/95	3/10	90/275	Non	Foreign
15 (3/2021)	Dakahlia	25/40	27/50	25/60	9/20	86/170	Non	Native
16 (12/2020)	Qalubia	19/70	16/60	20/85	5/15	60/230	Non	Native
17 (7/2021)	Behira	17/60	13/40	22/50	3/10	55/160	Non	Foreign
18 (5/2022)	Assiut	45/60	66/80	40/75	19/40	170/255	Non	Foreign
19 (5/2022)	Sohag	5/50	7/40	30/60	15/30	57/180	Non	Native

*Age of suckling rabbits was 17 up to 35 days, weaning rabbits 35 up to 55 days, growing rabbits 55 days up to 4 months, and adult rabbits more than 4 months.

Table 2. Molecular identified strains with their accession numbers

Serial No.	Governorate	Genotype	GenBank Accession No.
2	Kafr Elsheikh	RHDV2	OP716872
3	Kafr Elsheikh	RHDV2	OP716873
11	Damietta	RHDV2	OP716874
19	Sohag	RHDV1	OP716875

The sequences of Kafr Elsheikh (2019 and 2020) compared to each other revealed that the two strains were closely related to each other with an identity percentage of 100% and isolated from rabbits in Mahala (2019), Desouk (2019), Mit Ghamer (2019), and Benha (2019) with nucleotide identity percentages of 99.8%, 99.6%, 99.4%, and 99.2%, respectively. Little differences of these two strains were observed when compared to Damietta

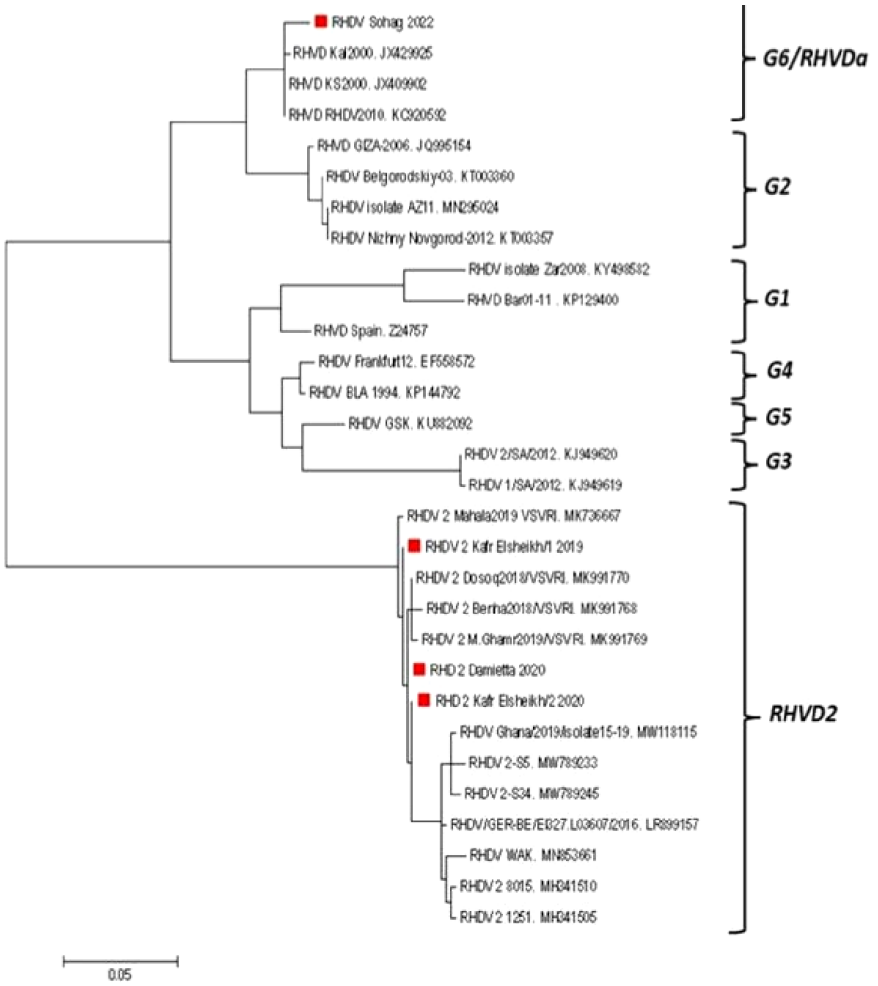

Fig. 1. Phylogenetic tree of 4 Egyptian RHDV isolates (Kafr Elsheikh 2019 and 2020, Damietta 2020, and Sohag 2022) constructed with other homologous and heterologous isolates retrieved from GenBank.

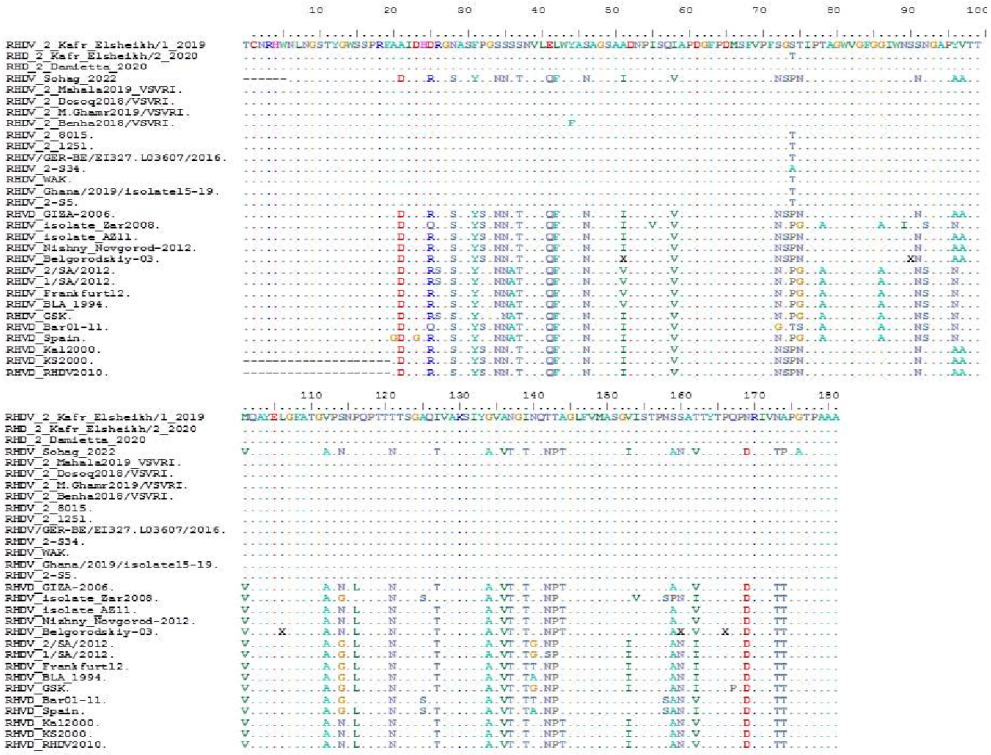

Fig. 2. Amino acids sequences of the characterised RHDV strains compared to other strains retrieved from Genbank database. Dots indicate identical sequence.

strain with an identity percentage of 99.999% (Fig. 2).

DISCUSSION

Continuous surveillance and monitoring of RHDV strains circulating in Egypt and updating the vaccinal strains are crucial issues. Hence, the current study aimed to identify and characterise RHDV in naturally infected rabbits and to assess the genetic diversity of RHDV circulating in different Egyptian provinces. Moreover, it aimed to determine the genetic relationship among such isolates at the molecular level from January 2019 to January 2022.

In the current study, data reported in Table 1 revealed that RHDV can cause higher mortality in suckling rabbit kittens compared to adults. Deaths are not common in rabbits less than 4 weeks old, possibly due to the age susceptibility or resistance (Elsworth *et al*., 2014). RHDV virulence depends on some factors associated with the virus as VP60 or host immunity (Calvete *et al*., 2018).

Rabbits naturally infected with Kafr Elsheikh and Damietta strains showed high mortalities of 90% and 62.5%, respectively. These results coincided with those obtained by Le Gall-Reculé *et al*. (2013), Dalton *et al*. (2018) and El-Samadony *et al.* (2021), who reported mortality rates of up to 50% for kittens involved in RHD mortality events caused by RHDV-GI.2. The clinical signs de-

tected in the affected farms agreed with those mentioned in OIE (2018) as nervous and respiratory signs, apathy and anorexia were observed.

PCR results approved that VP60 (C to E regions) could detect all RHDV genotypes as conserved primers (Embury-Hyatt *et al*., 2012), but with time benefit as the produced 600 bp fragment could be directly sequenced for genotyping suspected samples, particularly in the case of RHDV with negative HA activity (Abdel-Moaty *et al*., 2014).

As demonstrated by Le Gall-Reculé *et al*. (2013), sequence alignment studies of full-length VP60 of lagoviruses indicated that RHDV2 is a new genetic group, and it is phylogenetically distinct from all previous lagoviruses. Thus, the genetic relationships determined in previous studies confirmed that the sequence and phylogenetic analysis of the main antigenic determinant regions (C and E) of the *VP60* gene is reliable for genotyping RHDV (Moss *et al*., 2002; Le Gall-Reculé *et al*., 2003; Matiz *et al*., 2006; Le Gall-Reculé *et al*., 2011). It was also utilised for determining the genotype of circulating viruses in the Egyptian field (Abdel-Moaty *et al*., 2014; El-Bagoury *et al*., 2014).

The phylogenetic analysis results of 600 bp of hypervariable region of VP60 demonstrated that the three sequences of RHDV (Kafr Elsheikh 2019 and 2020, and Damietta strain) presented on Fig. 2 are clustered with other RHDV var 2 strain elsewhere in GenBank indicating that the three Egyptian strains belonged to RHDV var 2. Meanwhile, the Sohag (2022) isolate was grouped with RHDV var 1. Numerous authors agreed with the new classification of RHDV which proved the existence of the three main RHDV groups: classical, variant a, and variant 2 (Le Pendu *et al.,* 2017; Qi *et al.*, 2019; Abodalal & Tahoon, 2020; Erfan & Shalaby, 2020).

Blast analysis of the three Egyptian strain sequences compared to the other homologous RHDV var 2 Egyptian strains in GenBank evidenced that Kafr Elsheikh (2019 and 2020) and Damietta strains are closely related to other Egyptian strains isolated from rabbits in Mahala (2019), Desouk (2019), Mit Ghamer (2019), and Benha (2019) with nucleotide identity percentages of 99.8%, 99.6%, 99.4%, and 99.2%, respectively. Meanwhile, the Sohag (2022) isolate is closely related to Egyptian strains isolated from rabbits RHVD Giza (2006), RHVD Kal (2000), and RHVD KS (2000) with nucleotide identity percentages of 90.9%, 95.5%, and 91%, respectively. These findings agreed with Abodalal *et al*. (2021) who reported that several RHDV1 variant strains were detected and confirmed in multiple regions of Upper Egypt in 2019, posing a threat to the rabbit population. The rising question is whether the GI.2 strains will replace both classical and variant RHDV in Egyptian rabbit farms or all these genotypes will circulate together concurrently. RHDV2 has been reported to replace RHDV in rabbit population in south-western Europe (Le Gall-Reculé *et al*., 2013; Dalton *et al.,* 2014; Calvete *et al*., 2018; Lopes *et al.,* 2019). Thus, the epidemiological situation needs re-evaluation in Egyptian rabbit farms in order to determine the dominant RHDV genotype.

Recombination is a common feature of rabbit lagoviruses that may increase their genetic diversity and drive their evolution (Lopes *et al*., 2019; Hu *et al.,* 2021). Recombinant RHDV-G1/RHDV2 strains in rabbits and hares could be isolated in south-western France (Le Gall-Reculé *et al*., 2017). Thus, it is recommended to make full length sequence of *VP60* gene

for detecting any recombination events in recent Egyptian RHDV2 isolates in order to better understand virus evolution.

Regarding the different existing antigenic profile and genetic characteristics, the cross-protection between RHDV1 and RHDV2 is partial (Silvério *et al.,* 2018). These marked antigenic differences between both genotypes may cause the lack of efficient protection against RHDV2 afforded by the current RHDV inactivated vaccines (Le Gall-Reculé *et al*., 2013; Puggioni *et al*., 2013), which requires vaccinating rabbit farms by a vaccine containing two RHDV strains (RHDV1 and RHDV2) for protection against RHDV outbreaks.

CONCLUSION

The current study reported that the RHDV1 and RHDV2 circulating in Egypt cause high mortality in rabbit farms. These recent 4 isolates represent some Egyptian governorates (Delta and Upper Egypt). Continuous surveillance and monitoring of RHDV strains circulating in Egypt and updating the vaccine strains remains of crucial importance.

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