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RESEARCH

Genetic characterization of rabbit hemorrhagic disease virus from naturally-infected rabbits in Sharkia governorate, Egypt

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ABSTRACT

Background: Since the first introduction of rabbit hemorrhagic disease virus (RHDV) in 1991, Egyptian authorities started a national vaccination program against the disease. However, RHDV clinical cases are still being reported.

Objectives: This study aimed to investigate genetic characterization of recent RHDV isolates from naturally-infected rabbits in Sharkia governorate, Egypt in 2015/2016.

Methods: A total of 24 clinical samples were investigated by real-time reverse transcription PCR (RRT-PCR). Hemagglutination analysis of RRT-PCR positive samples were performed, also these positive RRT-PCR samples were purified and directly partial sequenced for C-terminal VP60 gene.

Results: Only two samples were positive for partial C-terminal VP60 amplification. The first positive sample was collected from non-vaccinated young rabbit (45-day old) and designed here as SHAH 2015, whereas the second sample was collected from non-vaccinated adult dam (nine-month old) and designed as SHMK2016. Hemagglutination analysis using human red blood cells type O revealed that both isolates were non-hemagglutinating. Sequence analysis revealed that both isolates shared amino acid identity of 98.57 % and 95.71 % with each other and with commonly used vaccinal strain (RHDV-Giza 2006), respectively. Phylogenetic analysis revealed that both isolates clustered with the vaccine strain and isolate UT01 within genotype 6 RHDVa. Molecular analysis of antigenic regions of VP60 (part of E and F regions) revealed neither detection of recombination points nor sites under positive selection.

Conclusion: The obtained results indicate that non-hemagglutinating RHDVa G6 variant viruses are currently circulating in Sharkia with the ability to infect both young and adult rabbits that may be due to inadequate application of vaccine and not due to vaccine mismatch. We recommend broad application of currently applied vaccine should include both young and adult rabbits.

Key words: RHDV, VP60, vaccination, recombination**,** RRT-PCR**,** Egypt.

BACKGROUND

Rabbit hemorrhagic disease (RHD) is a highly contagious and fatal disease for both wild and domestic rabbits (Oryctolagus Cuniculus) and it was first reported in China in 1984 (Liu *et al.*, 1984). The disease spread rapidly to Asia and Europe, and became endemic within a few years (Abrantes *et al.*, 2012, Alda *et al.*, 2010 and Le Gall Recule *et al.*, 2003). The RHD is caused by rabbit hemorrhagic disease virus (RHDV) which belongs to genus Lagovirus of the family Caliciviridae. RHDV is a non-enveloped single-stranded (ss) RNA genome organized into two narrowly overlapping open reading frames (ORFs). ORF1 includes nucleotide residues 10–7044 and encodes a large polyprotein which is cleaved by a virus-encoded protease to generate several non-structural (NS) proteins and the major capsid protein, VP60. ORF2 comprises nucleotide residues 7025–7378 and produces VP10, a minor structural protein (Du, 1991 and Wirblich *et al.*, 1996). At the 59 region, the RHDV genome presents a covalently linked protein, VPg, and is polyadenylated at the 39 region (Gregg *et al.*, 1991 and Morales *et al.*, 2004). In 1998 and 1999, the first reports describing a new RHDV variant (RHDVa) in Italy and Germany were published (Capucci *et al.*, 1998 and Schirrmeier *et al.*, 1999). Phylogenetic analysis of pathogenic RHDV strains shows three distinct groups: ‘‘classical’’ RHDV with the genogroups

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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). In Egypt, RHDV outbreak was firstly reported in Sharkia governorate in 1991 (Ghanem and Ismail, 1992). Subsequent outbreaks occurred in other governorates such as Kaluobia (Sharawi, 1992), Assuit (Salem and El-Ballal, 1992), Minia and Sohag governorates (El-Zanaty, 1994). For initial infection control, inactivated formalized tissue vaccine was firstly introduced (Salem and El-Zanaty, 1992). Other trials such as an inactivated formalized RHDV vaccine from the local isolate of RHDV (Egypt 96) adjuvanted with aluminum hydroxide and also rabbit pasteurellosis -RHDV combined vaccines were also used. (Daoud *et al*., 1998a, Daoud *et al.*, 1998b and Abd El-Motelib *et al*., 1998). Although using the intensive RHDV vaccination program, sporadic outbreaks were recorded in vaccinated flocks (Metwally and Madbouly,2005, Abd El-Lateff, 2006, Ewees,2007 and El-Sissi and Gafer, 2008). Moreover, circulation of variant RHDV strains have been detected in Egypt since 2007 (Ewees, 2007 and El-Sissi, Gafer, 2008 and Abd El-Moaty *et al.*, 2014) and these variant strains caused high morbidity and mortality with similar: clinical symptoms, Post-mortem (PM) and pathogenicity to that of the classical RHDV outbreaks, but the isolated virus lacked hemagglutinating (HA) activity. This study aimed to do a comprehensive analysis of recently isolated RHDV strains from Sharkia governorate, Egypt.

MATERIALS AND METHODS

Samples collection and preparation

A total of 24 clinical cases from eight different rabbitries in Sharkia governorate: Minia EL-Kamh (3 rabbitries), Abou-Hammad (3 rabbitries) and Belbeis (2 rabbitries) in the period of 2015 and 2016 were submitted to clinical and post-mortem examination at Veterinary diagnostic Unit, Avian and Rabbit Medicine Department, Zagazig University (Table 1). Affected rabbits were aseptically necrobized and three livers per farm were pooled and prepared into 10% suspension according to a standard protocol (OIE, 2010). The prepared suspensions were clarified by centrifugation at 3000 rpm for 15 minutes with chilling at 4˚C. The clear supernatants were collected and stored at -20˚C until use.

Table (1): Descriptive data of the examined rabbitaries.

Rabbiraty No.	Date of investigation	Locality	Total No.	Age	Mortality%	Vaccination
1	March 2015	Minia El Kamh	70	2ms	11	vaccinated
2	April 2015	Belbies	100	3.5ms	30	Not vaccinated
3	April 2015	Minia El Kamh	400	1.5ms	25	vaccinated
4	August 2015	Belbies	100	2ms	60	Not vaccinated
5	September 2015	Abou-Hammad	50	1.5ms	80	Not vaccinated*****
6	March 2016	Minia El Kamh	100	9ms	100	Not vaccinated
7	August 2016	Abou-Hammad	25	1ms	20	Not vaccinated
8	September 2016	Abou-Hammad	50	10ms	20	Vaccinated

*: owner vaccinated the dams only and sold the age between 2-3 months of age except few no. kept in the rabbitary without vaccination (this was source of infection).

Slide and micro-plate hemagglutination (HA) test

Positive RRT-PCR samples were tested for hemagglutination by slide and micro-plate HA tests. For slide HA, 100 µl of each isolate was mixed with 100 µl of 0.75% washed human red blood cells (RBCs) type O for one minute. For micro-plate HA, in brief, 50 µl of phosphate-buffered saline (PBS, pH 7.2) was added to all wells including positive and negative controls. Then 50 µl of prepared isolates was added to the first well only followed by twofold serial dilution. Finally, 50 µl of 0.75% washed human Red Blood Cells (RBCs) type O was added to all wells followed by incubation at 4°C for 1 hour.

Real-time reverse transcription reaction (RRT-PCR)

RNA was extracted from clarified supernatants using QIAamp RNeasy Mini kit (Qiagen, Germany, GmbH) according to the manufacturer's instructions. RRT-PCR was performed using specific primers (Metabion, Germany**)** for partial amplification of VP60 region c-terminal region according to (Fahmy et al., 2010) using Forward primer (P33): 5'CCA CCA CCA ACA CTT CAG GT'3 and reverse primer (P34): 5'CAG GTT GAA CAC GAG TGT GC '3 primers were utilized in a 25 µl reaction containing 12.5 µl of the 2x QuantiTect SYBR Green PCR Master Mix (Qiagen, Germany, GmbH), 0.25 µl of Revert Aid Reverse Transcriptase (200U/µL) (Thermo Fisher), 0.5 µl of each primer (20 pmol concentration each), 6.25 µl of water nuclease free, and 5 µl of extracted RNA. The reaction was performed in a MX3005P real time PCR machine (Agilent, USA) with the following cycling conditions: RT reaction for one cycle at 50 °C/30 min, initial denaturation at 94 °C/5 min, followed by 40 cycles of: denaturation at 94°C /15 sec. annealing 56°C/3 sec. extension 72 °C/40 sec. and final extension at 72 °C/10 min with, hold at 4 °C.

Sequencing and phylogenetic analysis

RRT-PCR samples were loaded on 1% agarose (molecular biology grade). Positive RRT-PCR samples were purified and directly sequenced at Virus Biotechnology Unit, Abassia Institute for Animal Health, Cairo using BigDye terminator v3.1 cycle sequencing kit according to the manufacturer's instructions. Raw sequence data were manually edited using geneious pro. 7.0(http://www.geneious.com/).New and publically available VP60 sequences were aligned and used to build phylogenetic trees using neighbor-joining method, the Tamura-Nei model, and 1000 bootstrap replicates implemented in MEGA 7.0 (Kumar *et al.,* 2016) and assembled RHDV sequences were deposited to the GenBank database.

Estimation of recombination and positive selection

To assess whether new RHDV isolates underwent positive diversifying selection, the calculated non-synonymous/ synonymous (dN/dS) ratio at each codon in the alignment was assessed using Datamonkey, (http://www.datamonkey.org/), a webserver of the HyPhy package, using the best-fit substitution model as recommended (Delport et al., 2010 and Pond et al., 2005). The aligned sequences (number = 25) were firstly analyzed for recombination using single-likelihood ancestor counting' algorithm (SLAC) method implemented into the Datamonkey server before analysis for positive selection. Four different codon-based maximum-likelihood methods at posterior probabilities (p. p) of 0.05 with the mixed-effects model of evolution (MEME), the most conservative algorithm (SLAC), ''fixed effects likelihood'' (FEL) and internal FEL (IFEL) were used (Pond et al., 2005).

RESULTS

Clinical and post-mortem examination of the naturally-infected rabbits

The clinical examination of the affected rabbits revealed sudden deaths with frothy bloody exudate from nostrils (few rabbits), whereas other rabbits revealed dullness, depression, convulsion, fever with cyanosis of lips and nostrils, dyspnea accompanied by abdominal respiration. Hematurea and hemorrhagic vaginal discharge were also observed in some cases. The common PM findings were dead rabbits in opisotonous position with severe congestion and frothy exudate in whole trachea (Fig. 1A & B). Hemorrhagic trachitis as well as congested, edematous and hemorrhagic lungs were also found (Fig. 1 C). The liver appeared pale, yellowish brown in color with focal, lobular necrosis with scattered hemorrhage of variable sizes. (Fig. 1D). Urinary bladder appeared full-filled with engorged blood vessels (Fig. 1 E).

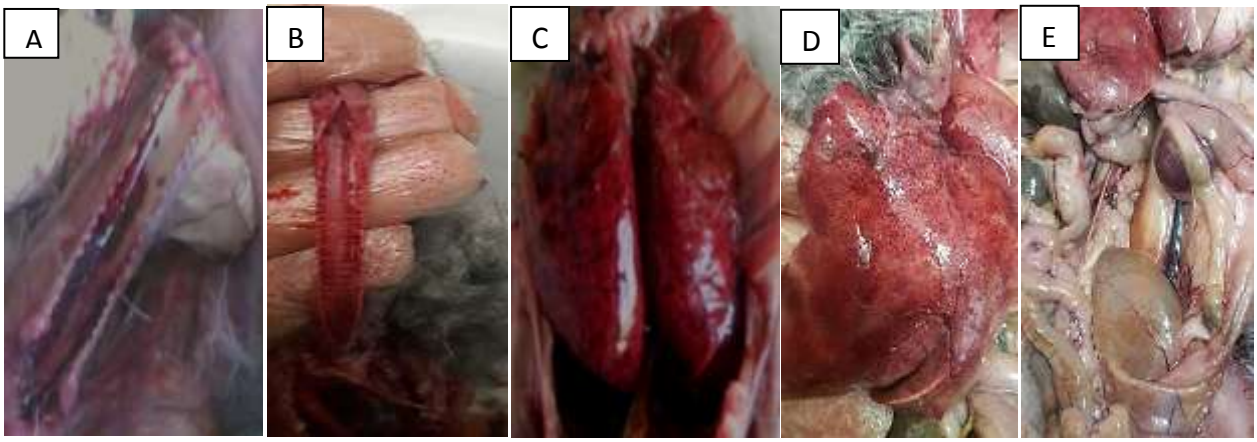

Fig. 1 PM lesions of naturally-infected rabbits.(**A** and **B**) Severe congestion and frothy exudate in whole trachea.(**C**) Diffuse congestion and hemorrhage on different lobes of lungs with free blood in thoracic cavity.(**D**) Hemorrhages of variable size (lobular pattern) on liver lobes. (**E**) Urinary bladder filled with discolored urine.

Three livers per farm were collected and prepared into 10% suspension under aseptic conditions. Clarified supernatants were divided into aliquots tested for hemagglutination against human RBCs type O either by slide and or micro-plate HA and RNA extraction. All clarified samples were negative for hemagglutination one hour post-incubation at 4 °C. All samples were tested in duplicate.

RRT-PCR and sequencing

Only two samples showed positive bands of correct size (538 bp) of VP60 C-terminal region against standard positive control. Sequenced isolates were designed as SHAH2015 (collected from non-vaccinated 45-day-old young rabbit) and SHMK2016 (collected from non-vaccinated nine-month-old dam) showed 98.57 % amino acid identity for each other. Moreover, both isolates showed 95.71 % amino acid identity with the commonly used vaccine strain (RHDV-Giza 2006, accession number JQ995154.1). Both isolates were submitted to GenBank with accession numbers (KY316900 & KY316901).

Phylogenetic and positive selection analysis

Clustal W logarithm implemented in geneious software was used to determine the alignment of the selected sequences. The phylogenetic tree was drawn using Mega 7.0 software with the neighboring joining model for calculation the genetic difference between sequences. The evolutionary distances were computed using the Maximum Composite Likelihood method. Phylogenetic analysis revealed that both sequences belongs to genotype 6 (RHDVa)) with the commonly used vaccine strain (**Fig 2**).

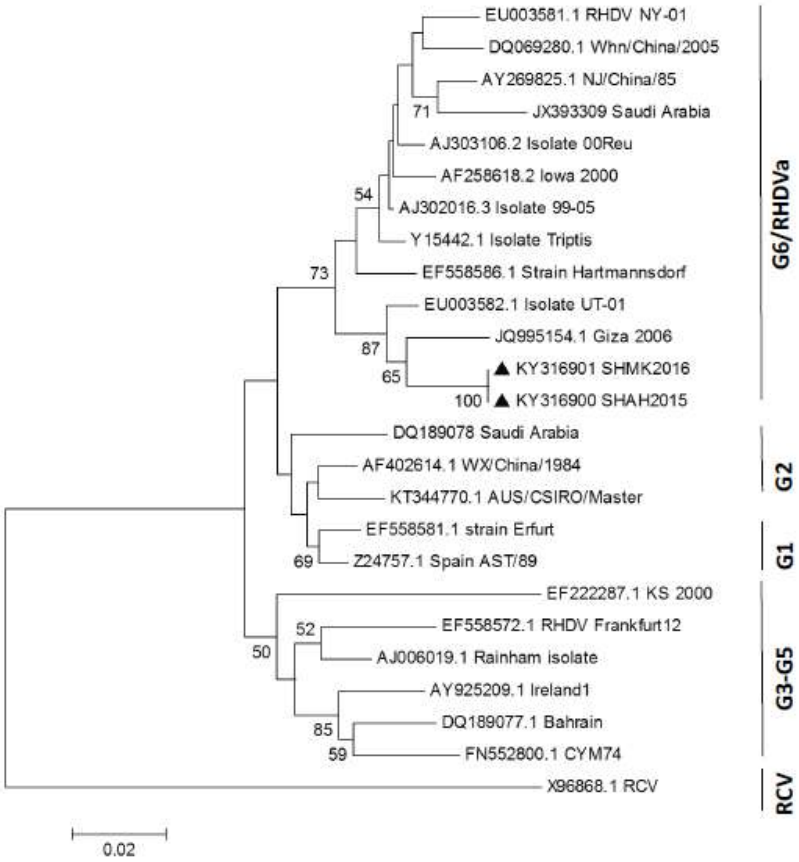

Fig. 2 Neighbor-joining phylogenetic tree of RHDV based on partial nucleotide sequences of the VP60 gene. Numbers at nodes represent the percentage of 1000 bootstrap replicates supporting the branch (values < 50% are not shown). The isolates from this study are indicated by *a solid triangle.*
Molecular analysis revealed neither recombination points in the aligned sequences nor sites under positive selection with 32 sites were detected under negative selection. To identify the most variable regions in RHDV VP60, the amino acid sequence was divided into six regions (A, B, C, D, E, and F) (OIE, 2010) and analyzed.

It was shown that RHDV VP60 protein (largely E and F regions) is informative for study of virus evolution (Pond et al., 2005). Informative regions are located in the C-terminal part of the VP60 gene and contain three previously identified positively selected codons (PSCs). Two positively selected codons (PSCs) at positions 432 in E region and 476 in F region were also analyzed. Molecular analysis revealed that there is little variation in antigenic regions (part of E and F regions) **(Fig 3).** There were12 amino acid mutations of the sequenced part of VP60 of the viruses with other reference strains published on Genbank.

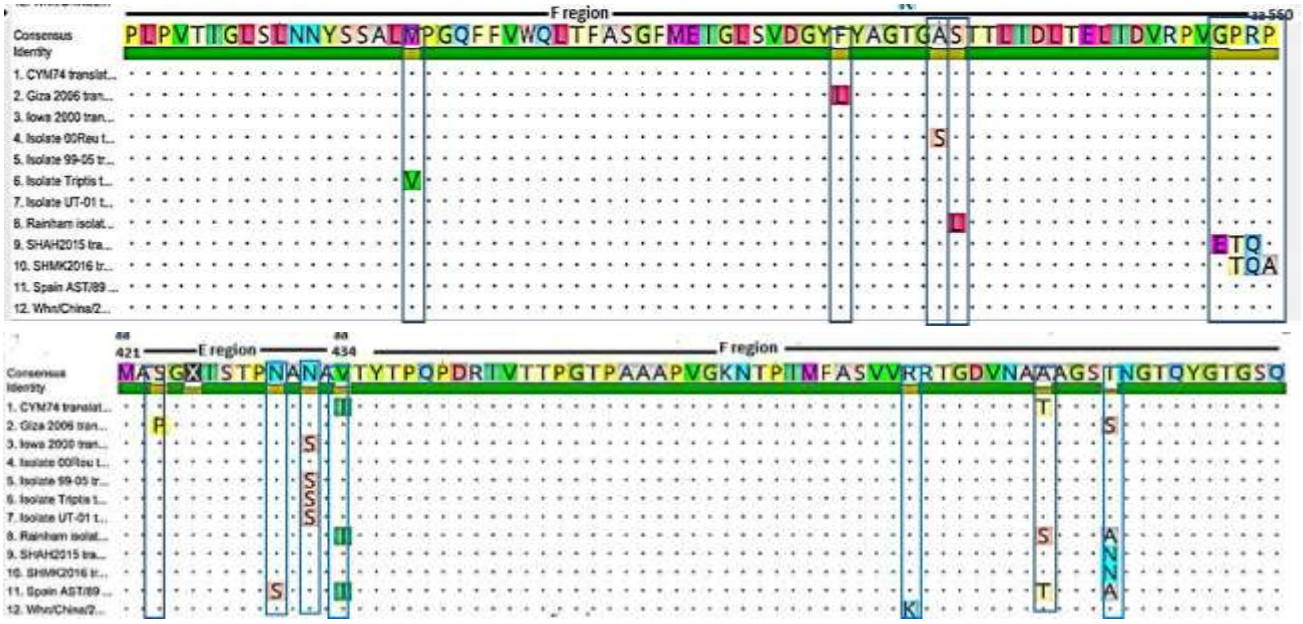

Fig. 3 Amino acid difference in antigenic regions of VP60 of E region (amino acids 421-434) and F region (435-560). Dots denote identical amino acids. Boxed segments indicate the amino acid substitution between our isolates of this study, vaccine strain (Giza 2006), and other reference strains. Black arrows indicate positively selected codons positions (PSCs) at E region (position 432) and F region (position 476).

DISCUSSION

The RHDV is the one of the most dangerous viral infections causes high mortalities and high economic losses annually for the industry of Rabbits in Egypt and worldwide. Despite intensive vaccination efforts to control RHDV infection in Egypt, clinical cases have been recorded recently from different localities in Sharkia governorate. Both RHDV isolates (SHAH2015, SHMK2016) showed acute disease clinical signs, which demonstrate their similarity in terms of virulence with previously reported natural RHDV outbreaks in Egypt. Both isolates were non-hemagglutinating against human RBCs type O, which may indicate current circulation of non-hemagglutinating RHDV strains in the Egyptian market. This is in agreement with previous reports (Abd El-Moaty et al., 2014 and Bazid et al., 2015) that recorded circulation of non-hemagglutinating RHDV in different regions in Egyt such as Munofia. Moreover, RHDV diagnosis depending on hemagglutination is not a reliable test as some isolates maybe non-hemagglutiating and other may show hemgglutination after passaging in susceptible rabbits as recorded by (Abd El-Moaty et al., 2014).

Genogroup G6 is the first antigenic variant of RHDV which was designated as RHDVa. Although this variant shares the same level of pathogeniciity as other RHDV viruses, it presented a distinct antigenic profile and genetic differences (Capucci et al., 1998). Interestingly, most of the amino acid variations found in RHDVa isolates were in region E (spanning the amino acid positions 344-370). These variants have been isolated in several countries and in some areas, they seem to be replacing the original strains (Mcintosh et al .,2007) which may be the case in Egypt. The partial VP60 sequence of both isolates showed 95.71 % amino acid identity with the common vaccinal strain (RHDV Giza 2006) and they clustered within the genotype 6. This indicates no diversity in the current circulating RHDV genotypes in different provinces of Egypt (Fig 4), where RHDV G6 and

G3-5 are currently circulating in different Egyptian governorates according to available sequence data. Moreover, old report reported RHDV outbreaks in other Egyptian provinces such as Cairo, Gharbia, Dakahlia, Beheira and Beni-Suef, but no sequence data is available to determine which genotype is currently circulating in these regions. On the other hand, recombination is not uncommon within the RHDV genome and might have played a role in the origin of the virus (Abrantes et al., 2008 and Forrester et al., 2008). However, our results showed no detection of recombination points in the aligned sequences and this might be due to lack of complete genome sequences.

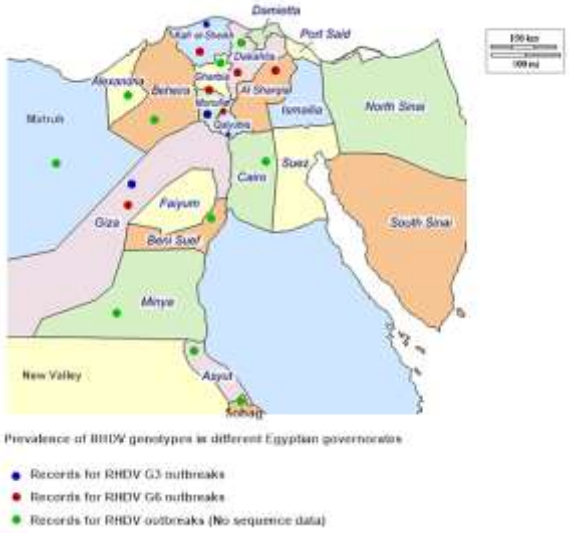

Fig. 4 Geographical distribution of RHDV strains across the Arab republic of Egypt between 1997 and 2016. A) Blue circles indicate RHDV G3-5 outbreak. Red circles indicate RHDV G6 outbreaks whereas green circles indicate RHDV outbreaks with unknown genotype due to lack of sequence data from these regions.

Although both isolates: SHAH2015 and SHMK2016 were isolated from rabbits of different ages, both sequences showed 98.57% amino acid identity with each other. This indicates that current RHDV outbreaks in Egypt relates to inadequate vaccine application and not vaccine mismatch. Three positively selected codons (PSCs) have been identified before at three of the six regions of the VP60 protein with two of them in E region (amino acid number 432) and F region (amino acid number 476) (Neill, 1992). Amino acid substitutions at the PSCs may impose changes in the polarity or charge of the protein, which are important for the protein structure and protein–protein interaction. Changes at the three PSCs can generate a putative N-glycosylation site (Esteves et al., 2008). N-linked glycosylation is one of post-translational modifications in newly synthesized proteins which allows viruses to increase surface diversity which affects infectivity, protein folding, tropism, proteolytic processing and immune evasion (Vigerust and Shepherd, 2007). Partial molecular analysis revealed neithe detection of recombination nor positive selection sites for our isolates. Moreover, both isolates showed no change at positions 432 and or 476, which may indicate that RHDV outbreaks in both young and adult rabbits is neither related to a change in N-glycosylation and nor to change in the transmissibility of the virus but due to inadequate application of vaccine. So, we recommend updating vaccination program to cover both young rabbits and old dams. Moreover, further

research about molecular evolution of RHDV in Egypt and antigenic characters of newly isolates should be assessed.

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