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Comparative Study between the Isolated Rabbit Hemorrhagic Septicemia Virus and Available Vaccine Strain

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ABSTRACT

Rabbit hemorrhagic disease virus (RHDV) is responsible for great economic losses in rabbit industry in Egypt. Several outbreaks of the disease were recorded along subsequent years until now despite of the availability of RHDV vaccine. The present study aimed to diagnose of RHDV from 31 suspected cases in addition to genetic comparison between recently isolated RHDVs and previously isolated viruses or available vaccine strain. Sixteen isolates were confirmed to be RHDV using haemagglutination test, animal inoculation and RT-PCR. Sequence and phylogenetic analysis for C-terminal VP60 gene of 7 isolates revealed the presence of newly emerged RHDV2 (Vet-Abotaleb, Vet-Alex-K15, Vet-Alex-B18, Vet-Alex-B26) in addition to classic RHDV Genotype 5 (Vet-Alex-Q2, Vet-Alex-Q7, Vet-Alex-K11). The nucleotide divergence between classic RHDV Genotype 5 to RHDV2 isolates ranged from 14.4-20.6%. Also, the newly isolated strains had nucleotide difference 11.2-21.2 % when compared to commonly vaccinal strain (RHDV-Giza 2006). Our findings indicated that there is amino acid substitution in S or A 476 E between the original RHDV isolates and vaccinal strain (Giza2006) to newly emerged RHDV isolates. The multiple alignment of deduced amino acid exhibited between the classic RHDV isolates and vaccinal strain to RHDV2 showed 13 constant amino acid differences at position I 551 V,V 553 I ,k 562 T ,E 549 D ,T 548 S,T 542 A, I 526 L,T 518 N,T 487 A ,S 476 E ,V 473 I ,V 455 I , D 441. It was concluded, the newly RHDV2 strain were isolated from suspected cases with still circulating classic RHDV in Egypt. There is high genetic diversity between isolates and vaccinal strain. So, the continuous and rapid evolution of those RHD viruses necessitates reviewing and updating for vaccine development.

Key words: Rabbit, RHDV, Haemagglutination test, RT-PCR, Sequencing, Evolution.

INTRODUCTION

Rabbit hemorrhagic disease is lethal viral infection of wild and domestic rabbits (Oryctolagus cuniculus) which caused by rabbit hemorrhagic disease virus (RHDV). RHDV belongs to family Caliciviridae, genus Lagovirus that is icosahedral, non-enveloped, single-stranded positive-sense RNA virus of approximately 7437 nucleotides with two open reading frames (Dalton *et al.*, 2015). Pathogenic RHDV strains are classified into three distinct groups: the "classic RHDV" with the genogroups G1–G5 isolated onwards, the antigenic variant RHDVa/G6, and RHDV2 (OIE, 2016).

The first known outbreak of RHD occurred in China in 1984 (Liu *et al.*, 1984), then it has rapidly spread to most parts of the world. Some classic RHDV strains lack of haemagglutination capacity (Kesy *et al.*, 1996). In 1996, a new variant, (RHDVa), was reported in rabbits in Italy and Germany (Capucci *et al.*, 1998), (Schirrmeier H *et al.*, 1999). A newly emerging RHDV called RHDV2 emerged in France in 2010, and has spread widely in France, Portugal, Australia and Sweden (Le Gall-Reculé *et al.*, 2013, Dalton *et al.*, 2014). In Egypt, the disease outbreak was introduced at 1991 in Sharkia province (Ghanem and Ismail. 1992), and then reported in several governorates (El-Mongy. 1998 and Mostafa. 2001) causing high morbidity and mortality of RHDV (Mohamed. 2009; Fahmy *et al.*, 2010). Variant RHDV was detected of in Egypt since 2007 (Ewees, 2007, El-Sissi and Gafer, 2008 and Abd El-Moaty *et al.*, 2014). Several outbreaks of the disease were recorded along subsequent years until now. Rabbits were infected by RHDV/RHDVa showed highest morbidity and mortality rates in adult rabbits but young rabbit less than 6–8 weeks old are less susceptible (Lavassa and Capucci, 2008) (OIE. 2016). The infection caused by RHDV2 infection have a variable mortality rate ranged (5–70%) with an average mortality of 20% in experimentally infected rabbits; death could occur in adult

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and lactating rabbits from 15 days of age. (Le Gall-Reculé *et al.*, 2011a, b) (OIE. 2016).

The hemagglutination test using human group O red blood cells was applied for routine laboratory diagnosis of RHDV but, it is not a reliable for the diagnosis of the RHD virus because non-hemagglutinating isolates of the virus *(*El-Bagoury *et al.*, 2014; Bazeid *et al.*, 2015). So, virus detection and characterization were exhibited by inoculation of susceptible rabbits, (OIE. 2012; Ismail *et al.*, 2017). The VP60 capsid protein is the main structural protein of RHDV which contains the type-specific antigenic epitope (Capucci *et al.*, 1998). Genetic variations between RHDV strains can be evaluated through phylogenetic studies based on partial and complete sequences of this gene (Tian *et al.*, 2007).

Vaccination is important tool for controlling the disease. Commercial vaccines are based on adjuvanted, inactivated virus isolated from the liver of experimentally infected rabbits (Salman. 2007). Despite of RHDV vaccination programs applied on rabbit farms, there is a continuous circulation of RHDV outbreak in Egypt (Elbagoury *et al.*, 2014). So, the present study was conducted to evaluate the recent circulated isolates of RHDV if present and genetic variability between these suspected isolates and that of commonly used Giza 2006 vaccine or the previously isolated RHDV strains.

MATERIALS AND METHODS

Reference virus

RHD virus was kindly supplied by gene back department in central laboratory of veterinary biologics which used by CLEVB in challenge test and in preparation of RHDD-HA antigen. The virus genome was used as a positive control for virus detection by conventional PCR assay.

Field samples

A total of 31 liver samples were collected from freshly dead rabbits raised in El-Qalubia, El-Behera and Kafr El Sheikh Governorates during the period 2018 and first half 2019. Prior to death, the rabbits displayed signs of low spirit, urgent breath, yelping and struggling motion, some rabbits hemorrhaged from nostrils. Necropsy findings showed characteristic pathological lesions including pale, fragile liver, often with accentuation of the lobular markings and interspersed with hemorrhages, an enlarged, congested spleen, reddish speckled kidneys and lungs with hemorrhagic lesions of different degrees. Data about number, locality and mortality rate of the infected rabbit farms were illustrated in Table 1.

Virus Isolation (OIE. 2018)

The collected infected livers were homogenized and 10 % of Liver suspension in phosphate-buffered saline (PBS) were prepared. The liver homogenates were clarified by centrifugation at 3000 rpm for 20 minutes, and then supernatant were collected and tested for viral haemagglutination activity by hemagglutination test (Salman *et al.*, 2010). Positive samples were inoculated into seronegative susceptible rabbits at the age of 3 months weighting 1.5 to 2 kg. Two rabbits were kept as non-infected controls. The mortality % was recorded with the HA activity for each strain (Ferreira *et al.*, 2004).

Table 1: Data of field liver samples Collected from infected rabbit farms.

Government	Town	Isolate code	Type of breed	Vaccination	Mortality %	
					Un-weaned	Adult
Qalyubia	Qalyub	Q1	Multiple breed	Vaccinated	Found	40%
		Q2	Baladi	Not vaccinated	Not found	60%
	Banha	Q3	California, New Zealand	Not vaccinated	Not found	40%
		Q4	New Zealand	Vaccinated	Not found	30%
	Shibin alqantar	Q5	Multiple breed	Vaccinated	Found	10%
	El-kanater alkhiria	Q6	Baladi	Vaccinated	Not found	30%
	Toukh	Q7	Multiple breed	Not vaccinated	found	70%
		Q8	Highplus	Vaccinated	Found	20%
		Q9	California, New Zealand	Vaccinated	Not found	60%
		Q10	California, New Zealand	Vaccinated	Found	45%
Kafr-Elsheikh	Baltim	K11	Multiple breed	Vaccinated	Not found	20%
		K12	Multiple breed	Vaccinated	Found	15%
		K13	Multiple breed	Not vaccinated	Found	40%
		K14	Baladi	Not vaccinated	found	30%
	Kafr-elsheik	K15	Multiple breed	Not Vaccinated	found	40%
		K16	Multiple breed	Vaccinated	Not found	15%
		K17	Highplus	Vaccinated	Found	85%
Beheira	Abo-Elmatamer	B18	Multiple breed	vaccinated	found	30%
		B19	Baladi	Not Vaccinated	Found	15%
		B20	New Zealandi	Vaccinated	Found	60%
	Damanhur	B21	Multiple breed	Not vaccinated	Found	15%
		B22	Multiple breed	Vaccinated	Not found	40%
Beheira		B23	Multiple breed	vaccinated	found	30%
		B24	Multiple breed	Vaccinated	Not found	15%
		B25	Baladi	Not Vaccinated	found	25%
		B26	Multiple breed	Not Vaccinated	found	50%
	El-mahmoudiyah	B27	California, New zealand	Vaccinated	found	30%
		B28	New Zelandi	Not vaccinated	found	40%
	Etay-Elbarud	B29	Baladi	vaccinated	Found	25%
		B30	Multiple breed	Not vaccinated	Found	30%
		B31	Baladi	Vaccinated	Not found	10%

Table 2: Primers sequences and RT-PCR reaction scheme.

Amplified gene	Primers sequences 5`---3` (reference)	RT-PCR reaction scheme						Product size
		RT	One cycle	35 cycles			Final Extension	
			Primary denaturation	Denaturation	Annealing	Extension		
VP60	F. CCACCACCA ACACTTCAGGT R. CAGGTTGAA CACGAGTGTGC	45C°/30 min	95°C/15 min	95°C /1min	56°C/1 min	72°C/2min	72°C/10 min.	538bp

Molecular identification of RHDV virus by conventional RT-PCR assay

RNA was extracted from clarified supernatants using QIAamp Viral RNA Mini Kit (QIAGEN) according the manufacturer's instructions. RT-PCR technique was done for the isolated RNAs using RT-PCR kit (Qiagen one step RT-PCR kit, QIAGEN Gmb H, Germany). A set of specific primers were supplied from (Biosearch tech.com) and used for amplification of VP60 region c-terminal region (Fahmy et al., 2010). The amplification reaction scheme was done as described in Table 2.

Sequencing and phylogenetic analysis

Positive RT PCR samples were purified by using QIAquick PCR Product extraction kit. (Qiagen Inc. Valencia CA) according the manufacturer's instructions and followed by sequencing using Big dye TerminatorV3.1 cycle sequencing kit (Perkin-Elmer, Foster city, CA) with the same primers used for RT-PCR according to the manufacturer's instructions in Applied Biosystems 3130 genetic analyzer (ABI, USA .A comparative analysis of sequences was performed using the CLUSTALW multiple sequence alignment program, version 1.83 of MegAlign module of Lasergene DNAStar software Pairwise, which was designed by Thompson *et al.,* (1994). Sequence alignments and phylogenetic comparisons of the aligned sequences for the gene were also performed with the MegAlign module of Lasergene DNAStar software to determine nucleotide and amino acid sequence similarities and relationships. The sequence analysis and comparison occurred between the sequences of locally isolated viruses in this study and sequences posted in gene bank for other RHDV isolates and standard vaccine strains were shown in Fig. 4.

RESULTS

Viral identification:

HAT was performed on re-isolated RDHV samples at the recommended temperature 4°C (OIE. 2016). The results presented in Table (3) revealed that 15 out of 31 tested viral samples were positive for haemagglutination test with titer values ranged from 5 to 13 log2.

Also, the data illustrated in Table (4) showed that, the experimentally inoculated rabbits with the re-isolated viral samples had the same clinical picture of natural RHDV infection with a mortality rate ranging from 20-80% .The control non-infected rabbits were alive without any symptoms. The results of HA test applied on viral samples after passage on susceptible rabbits were RHDV positive and the HA titers ranged from 5 to 14 log_2.

RT-PCR identification of RHDV

All isolated viral samples either HA positive or negative were confirmed for the presence of RHDV virus by vp60 gene –based RT-PCR assay.

It was found that, 16 viral samples out of 31 (Q2,Q3,Q4,Q5,Q6,Q7,Q9,K11,K13,K14,K15,B18,B25,B26,B28 and B29) were RT-PCR positive for RHVD as shown in Fig. (1, 2 and 3).

Sequencing and genetic analysis

The amplified VP60 gene sequences of the isolated RHDVs were selected from 16 positive RHVD isolates, according to their government of isolation, antigenicity, rabbit age susceptibility and from either vaccinated or unvaccinated farm, for sequence analysis with other RHVD strains. the chosen viral samples were named RHDV\Vet-Alex-Q2, RHDV\Vet- Abotaleb(Q5), RHDV\ Vet-Alex- Q7, RHDV\Vet-Alex-K11, RHDV\Vet-Alex-

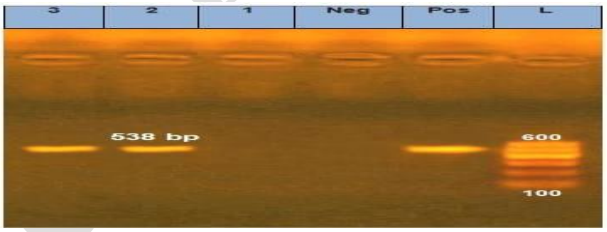

Fig. 1: shows RT-PCR of RHVD-VP_{60} gene. Lane 1: 100 bp DNA ladder, Lane 2: positive control, Lane 3: negative control, Lane 4: sample Q1 (negative), lane 5&6: samples Q2 and Q3 (positive).

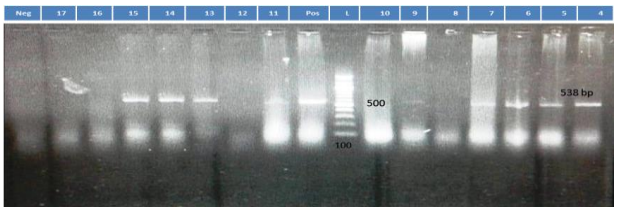

Fig. 2: shows RT-PCR of RHVD-VP_{60} gene. Lane 8: 100 bp DNA ladder, Lane 9: positive control , Lane 18: negative control, Lane 5, 7, 11, 15 & 16: sample Q1 (negative), Lane 1, 2, 3, 4, 6, 10, 12, 13&14: samples Q4, Q5, Q6, Q7, Q9, K11, K13, K14 and K15 (positive).

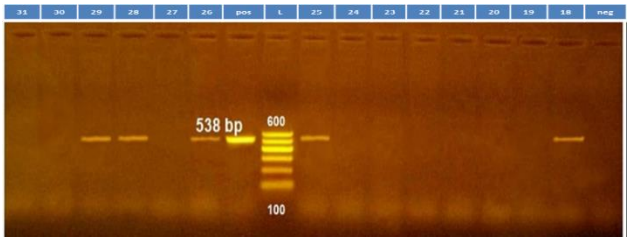

Fig. 3: shows RT-PCR of RHVD-VP_{60} gene. Lane 10: 100 bp DNA ladder, Lane 11: positive control, Lane 1: negative control, Lane 3, 4, 5, 6, 7, 8, 13, 16 & 17: samples B19, B20, B 21, B 22, B 23, B 24, B 27, B 30 and B 31 (negative), Lane 2, 9, 12, 14 &15: samples B18, B 25, B 26, B28 and B29 (positive).

Table 3: HA titers of the tested viral samples isolated from infected rabbits using human type O erythrocytes.

viral samples code	Slide HAT result	viral HA titer by microtiter HAT
Q2	+ve	2^{13}
Q3	+ve	2^{8}
Q5	+ve	2^{12}
Q6	+ve	2^{7}
Q7	+ve	2^{11}
Q9	+ve	2^{10}
K11	+ve	2^{12}
K13	+ve	2^{10}
K14	+ve	2^{5}
K15	+ve	2^{11}
B18	+ve	2^{11}
B25	+ve	2^{8}
B26	+ve	2^{12}
B28	+ve	2^{10}
B29	+ve	2^{11}

Table 4: Results of virus inoculation in experimental rabbits.

Viral samples code	No of deaths in experimental rabbit till 7 days after inoculation	Mortality %	Slide HAT result	Viral HA titer by microtiter HAT
Q2	4/5	80%	+ve	2^{14}
Q3	2/5	40%	+ve	2^{10}
Q4	3/5	60%	-ve	0
Q5	1/5	20%	+ve	2^{13}
Q6	1/5	20%	+ve	2^{9}
Q7	3/5	60%	+ve	2^{12}
Q9	3/5	60%	+ve	2^{11}
K11	3/5	90%	+ve	2^{12}
K13	2/5	40%	+ve	2^{11}
K14	1/5	20%	+ve	2^{5}
K15	2/5	40%	+ve	2^{13}
B18	1/5	20%	+ve	2^{12}
B25	1/5	20%	+ve	2^{10}
B26	2/5	40%	+ve	2^{13}
B28	1/5	20%	+ve	2^{11}
B29	1/5	20%	+ve	2^{11}

K15. RHDV\Vet-Alex-B18 and RHDV\Vet-Alex-B26 with the accession numbers MN276175, MN276176, MN276179, MN276180, MN276181, MN276177 and MN276178 as shown in (Table 5).

It was shown from the phylogenetic tree (Fig. 4) that all RHVD strains were subdivided into two groups, the first one was represent the classic RHD genotype 5 (French RHDV strain 01-15=CYM74, Italian RHDV strain Or08, RHDV 00-13 and French RHDV strain Ca11) and the second one contain the RHDV2 (Italian RHDV2strain RHDV Vs11_1 as well as the RHDV Vs11_2 , RHDV Vs11, RHDV Ss11).

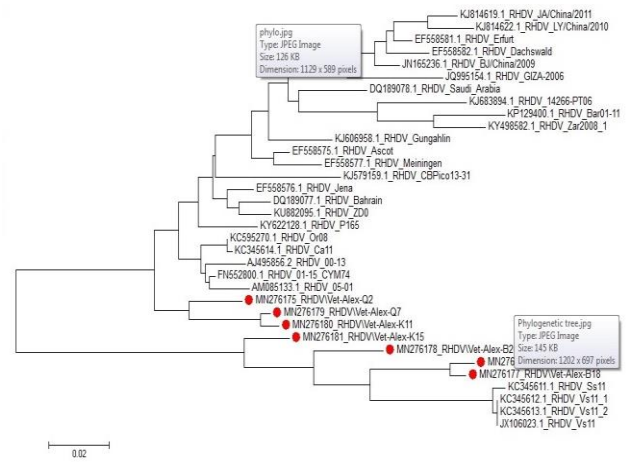

Fig. 4: phylogenetic tree of RHDV based on partial nucleotide sequences of the VP60 gene. The isolates from this study are indicated by a circle.

In comparison between the reference strains available in the GenBank database and submitted isolates, it was found that, four RHDV samples was related to the RHDV2 strains group (RHDV\Vet-Abotaleb, RHDV\Vet-Alex-K15 , RHDV\Vet-Alex-B18 and RHDV\Vet-Alex-B26) with homology percentage of 89.9 – 92.9%. While, the other three isolates RHDV\Vet-Alex-Q2, RHDV\Vet-Alex-Q7 and RHDV\Vet-Alex-K11 were fell in the classic RHDV genotype 5 group with homology percent of 93 – 96% as shown in Fig. 4, Table 5 and 6.

On other side, it was found that local isolates RHDV\Vet-Alex-Q7 and RHDV\Vet-Alex-K11 showed 99.1 % identity with each other on nucleotide sequence but on amino acid sequence level showed 99.4%. While RHDV\Vet-Alex-Q2 exhibit 95.7-95.5% with the previous isolates, respectively on nucleotide sequence level as well as 97.7%-98.3% on amino acid level. (Fig. 5 and Table 6). Also, the second group isolates of RHDV\Vet-Abotaleb showed 98.7% homology with RHDV\Vet-Alex-B18, 95.9 % with RHDV\Vet-Alex-B26 and 92.7% with RHDV\Vet-Alex-K15 on the level of nucleotide similarity. While, amino acid similarity within the second group ranged from 91.5%-99.4%. This means that, The divergence percentage between classical Egyptian isolates was very low (0.9 – 4.5%) and variability was 1.3-7.3 % within newly emerged RHDV2. On parallel to, it showed that, the nucleotide similarity between the two groups of the isolated RHDV is ranged from 79.4%-85.6% with variability value was 14.4 up to 20.6% on the nucleotide sequence level. On the other hand, the result of alignment between current isolates and vaccinal strain of (RHDV GIZA-2006) exhibited similarity ranged from 78.8 to 88.8% as shown in Table 6.

Table 5: Database of the different RHDV isolates on Genbank.

Government	Isolate number	Strains name	Accession no.	Genotype
Qalubia	Q2	RHDV.sqn RHDV\Vet-Alex-Q2	MN276175	RHDV (genotype 5)
	Q5	RHDV.sqn RHDV\Vet-Abotaleb	MN276176	RHDV2
	Q7	RHDV.sqn RHDV\Vet-Alex-Q7	MN276179	RHDV (genotype 5)
K afr-Elshiekh	K11	RHDV.sqn RHDV\Vet-Alex-K11	MN276180	RHDV (genotype 5)
	K15	RHDV.sqn RHDV\Vet-Alex-K15	MN276181	RHDV2
Bahiera	B18	RHDV.sqn RHDV\Vet-Alex-B18	MN276177	RHDV2
	B26	RHDV.sqn RHDV\Vet-Alex-B26	MN276178	RHDV2

Table 6: Results of sequence identity Matrix of the present RHDV isolates and other published available sequence on Genbank

Percent Identity

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	
1		90.4	98.9	98.7	98.3	98.5	79.0	79.2	79.2	79.2	96.3	96.6	96.3	93.8	95.5	94.2	94.2	92.9	91.6	89.0	90.1	96.1	89.7	96.1	80.3	80.7	83.1	94.8	94.2	86.7	1
2	10.5		90.4	90.3	89.9	89.1	79.0	79.2	79.2	79.2	90.8	91.4	91.2	90.3	92.3	92.7	91.4	92.5	95.3	89.0	88.8	91.2	93.1	88.4	78.8	79.2	80.5	87.6	87.6	82.0	2
3	1.1	10.5		99.8	97.6	97.6	79.6	79.8	79.8	79.8	95.9	95.7	95.5	93.4	94.8	93.4	93.8	92.1	91.2	88.6	89.7	95.3	90.1	95.7	80.1	80.5	83.1	94.0	93.4	86.7	3
4	1.3	10.7	0.2		97.4	97.4	79.8	80.0	80.0	80.0	95.7	95.5	95.3	93.3	94.6	93.3	93.6	91.9	91.0	88.4	89.5	95.1	89.9	95.5	80.3	80.7	83.3	93.8	93.3	86.9	4
5	1.7	11.1	2.5	2.7		97.4	78.7	78.8	78.8	78.8	95.3	95.7	95.3	93.1	94.6	93.3	93.6	92.5	91.0	88.6	89.0	95.1	89.1	95.7	79.6	80.0	82.4	94.4	93.8	86.0	5
6	1.5	12.1	2.5	2.7	2.7		78.5	78.7	78.7	78.7	95.1	95.3	94.9	92.9	94.2	92.9	93.3	91.9	90.6	88.0	89.1	94.8	89.0	94.6	79.6	80.0	82.4	94.0	93.4	85.6	6
7	25.5	25.5	24.6	24.3	26.1	26.3		99.4	99.4	99.4	79.4	79.4	79.6	78.7	80.0	79.4	80.9	79.6	79.2	79.6	79.2	79.2	80.9	78.1	92.9	92.7	89.9	79.0	78.7	87.6	7
8	25.2	25.2	24.3	24.0	25.8	26.0	0.6		100.0	100.0	79.6	79.6	79.8	78.8	80.1	79.6	81.1	79.8	79.4	79.8	79.0	79.4	81.1	78.3	93.1	92.5	90.1	79.2	79.2	87.8	8
9	25.2	25.2	24.3	24.0	25.8	26.0	0.6	0.0		100.0	79.6	79.6	79.8	78.8	80.1	79.6	81.1	79.8	79.4	79.8	79.0	79.4	81.1	78.3	93.1	92.5	90.1	79.2	79.2	87.8	9
10	25.2	25.2	24.3	24.0	25.8	26.0	0.6	0.0	0.0		79.6	79.6	79.8	78.8	80.1	79.6	81.1	79.8	79.4	79.8	79.0	79.4	81.1	78.3	93.1	92.5	90.1	79.2	79.2	87.8	10
11	3.9	10.0	4.3	4.5	4.9	5.1	24.9	24.6	24.6	24.6		97.0	95.7	93.1	95.1	94.0	93.8	93.6	91.9	88.8	90.3	96.1	89.7	93.8	80.7	81.1	83.3	92.9	92.7	86.5	11
12	3.5	9.3	4.5	4.7	4.5	4.9	24.8	24.5	24.5	24.5	3.1		97.8	94.4	96.8	94.9	95.5	93.6	92.5	89.3	90.4	97.8	90.6	93.6	80.1	80.5	82.4	92.5	92.3	85.8	12
13	3.9	9.5	4.7	4.9	4.9	5.3	24.5	24.2	24.2	24.2	4.5	2.3		94.0	96.1	94.6	94.8	92.9	92.3	89.3	90.1	97.9	90.8	92.9	80.1	80.5	82.0	91.4	91.2	85.4	13
14	6.6	10.7	7.0	7.2	7.4	7.6	26.0	25.7	25.7	25.7	7.4	5.9	6.3		93.4	92.3	92.9	91.4	91.0	87.5	88.6	94.2	89.7	91.9	78.8	79.0	81.3	90.3	89.7	83.9	14
15	4.7	8.2	5.5	5.7	5.7	6.1	24.0	23.7	23.7	23.7	5.1	3.3	4.1	7.0		96.6	97.9	95.1	93.8	90.6	92.5	96.4	91.9	91.9	80.0	80.3	81.6	91.8	91.6	84.3	15
16	6.1	7.8	7.0	7.2	7.2	7.6	24.9	24.6	24.6	24.6	6.3	5.3	5.7	8.2	3.5		95.3	94.9	93.3	90.6	92.7	94.6	91.6	91.6	80.0	80.3	81.8	90.4	90.3	83.7	16
17	6.1	9.3	6.6	6.8	6.8	7.2	22.6	22.4	22.4	22.4	6.5	4.7	5.5	7.6	2.1	4.9		94.0	92.9	89.7	91.8	95.1	92.1	90.6	80.7	81.3	82.0	90.8	90.6	84.3	17
18	7.6	8.1	8.5	8.7	8.0	8.7	24.6	24.3	24.3	24.3	6.7	6.7	7.6	9.4	5.1	5.3	6.3		94.8	91.4	93.1	93.6	92.1	90.6	79.0	79.4	80.1	90.6	90.1	82.8	18
19	9.2	4.9	9.6	9.8	9.8	10.3	25.3	25.0	25.0	25.0	8.7	8.0	8.2	9.8	6.5	7.2	7.6	5.5		90.4	91.0	92.3	95.9	89.5	79.8	80.0	81.1	89.5	89.5	82.6	19
20	12.2	12.2	12.7	12.9	12.7	13.4	24.4	24.1	24.1	24.1	12.4	11.7	11.7	14.1	10.1	10.2	11.3	9.3	10.4		93.6	89.0	88.8	86.9	78.5	78.8	79.4	87.1	86.5	80.9	20
21	10.8	12.5	11.3	11.5	12.2	12.0	25.0	25.3	25.3	25.3	10.6	10.4	10.8	12.7	8.0	7.8	8.8	7.4	9.7	6.7		90.1	89.7	87.1	79.6	80.0	80.5	88.0	87.8	81.3	21
22	4.1	9.5	4.9	5.1	5.1	5.5	25.1	24.9	24.9	24.9	4.1	2.3	2.1	6.1	3.7	5.7	5.1	6.7	8.3	12.2	10.8		90.4	93.3	79.4	79.8	81.3	91.9	91.8	84.3	22
23	11.4	7.4	10.9	11.2	12.1	12.3	22.6	22.4	22.4	22.4	11.4	10.2	10.0	11.4	8.7	9.2	8.4	8.5	4.3	12.4	11.3	10.5		87.6	80.3	80.5	81.5	87.8	88.0	82.4	23
24	4.1	12.9	4.5	4.7	4.5	5.7	26.8	26.5	26.5	26.5	6.5	6.7	7.6	8.7	8.7	9.0	10.2	10.2	11.6	14.8	14.6	7.2	13.9		79.4	79.8	83.3	95.7	95.5	86.0	24
25	23.5	25.8	23.7	23.5	24.6	24.6	7.6	7.4	7.4	7.4	22.9	23.6	23.7	25.7	24.0	24.0	22.9	25.5	24.4	26.2	24.5	24.9	23.5	24.7		98.7	95.9	80.7	80.7	92.7	25
26	22.9	25.2	23.2	22.9	24.0	24.0	7.8	8.0	8.0	8.0	22.3	23.1	23.1	25.4	23.4	23.4	22.0	24.9	24.1	25.5	23.9	24.2	23.2	24.1	1.3		94.6	81.1	80.7	91.8	26
27	19.6	23.5	19.6	19.3	20.7	20.6	11.1	10.8	10.8	10.8	19.3	20.6	21.1	22.3	21.7	21.5	21.2	24.0	22.7	25.0	23.3	22.3	22.0	19.3	4.2	5.7		83.7	83.7	95.7	27
28	5.5	13.9	6.3	6.5	5.9	6.3	25.4	25.1	25.1	25.1	7.6	8.0	9.3	10.7	8.9	10.4	10.0	10.2	11.6	14.6	13.4	8.7	13.7	4.5	22.8	22.2	18.8		99.1	85.6	28
29	6.1	13.8	6.9	7.2	6.5	6.9	25.9	25.0	25.0	25.0	7.8	8.2	9.5	11.4	9.1	10.6	10.2	10.8	11.5	15.2	13.6	8.9	13.4	4.7	22.8	22.7	18.7	0.9		85.6	29
30	14.9	21.3	14.9	14.7	15.9	16.4	13.8	13.6	13.6	13.6	15.2	16.1	16.6	18.7	18.1	18.9	18.2	20.2	20.6	22.8	22.3	18.1	20.8	15.9	7.7	8.8	4.5	16.4	16.3		30
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	

Divergence

1 FN552800.1 RHDV 01-15=CYM74
2 JQ995154.1 RHDV GIZA-2006
3 KC595270.1 RHDV Or08
4 KC345614.1 RHDV Ca11
5 AJ495856.2 RHDV 00-13
6 AM085133.1 RHDV 05-01
7 KC345611.1 RHDV Ss11
8 KC345612.1 RHDV Vs11_1
9 KC345613.1 RHDV Vs11_2
10 JX106023.1 RHDV Vs11
11 KY622128.1 RHDV P165
12 EF558576.1 RHDV Jena
13 DQ189077.1 RHDV Bahrain
14 KJ579159.1 RHDV CBPico13-31
15 EF558575.1 RHDV Ascot
16 KJ606958.1 RHDV Gungahlin
17 EF558577.1 RHDV Meiningen
18 DQ189078.1 RHDV Saudi Arabia
19 JN165236.1 RHDV BJ/China/2009
20 KP129400.1 RHDV Bar01-11
21 KJ683894.1 RHDV 14266-PT06
22 KU882095.1 RHDV ZD0
23 KJ814619.1 RHDV JA/China/2011
24 MN276175 RHDV\Vet-Alex-Q2
25 MN276176 RHDV\Vet-Abotaleb
26 MN276177 RHDV\Vet-Alex-B18
27 MN276178 RHDV\Vet-Alex-B26
28 MN276179 RHDV\Vet-Alex-Q7
29 MN276180 RHDV\Vet-Alex-K11
30 MN276181 RHDV\Vet-Alex-K15

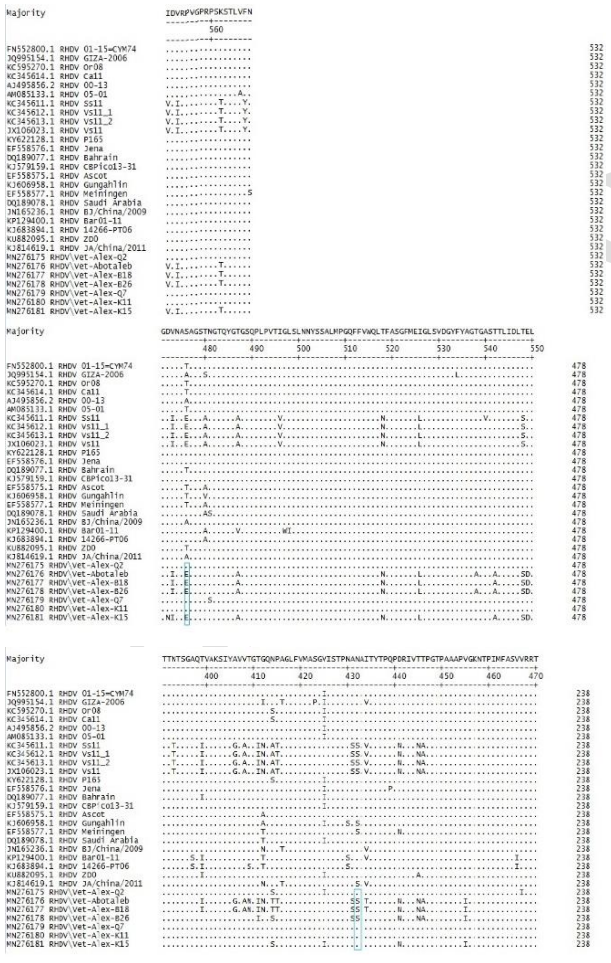

Fig. 5: Amino acid sequences of RHDV isolates, reference Strains and vaccine strain (Giza 2006) on the gene bank. Dots denote identical Amino acid sequence. Blue boxes indicate positively selected codons positions (PSCs) at E region (position 432) and F region (position 476).

DISCUSSION

In Egypt, RHDV infection is still representing a threat for the rabbit production farms in spite of vaccination pro-grams (Ewees, 2007; El-Bagoury *et al.*, 2014). So, updating the current situation is very important to find the key tool for resolving this problem and establish the new strategy to face of that disease.

Monitoring and preliminary diagnosis of RHDV from 31 outbreaks from suspected cases were done during 2018 and first half 2019 in 3 different governments (El-Qalubia, El-Behera and Kafr El Sheikh). Virus isolation from that infected farms and comparative molecular identification to other isolated RHDVs were done.

The data revealed from Table 1 showed the collected liver samples were actually representative and reflect the real picture for this disease because they include different rabbit ages, breeds, mortality rates, breeding sector and vaccination states of suspected cases.

Liver samples were tested by HA test at 4°C (Capucci *et al.*, 1991 and OIE. 2016). From Table 2, 15 out of 31 of suspected samples were able to agglutinate human type (O) RBCs with HA titers ranging from 2^5 to 2^{13} which proved by Le Gall-Reculé *et al.*, 2013 and Ewees. 2007 that said RHDV isolates agglutinates human RBC of type "O" efficiently.

The reverse transcription (RT)-PCR was applied for detection of RHDV-specific nucleic acid (Gould *et al.*, 1997). it was revealed that 16 out of 31 viral samples were positive (Table 3). This means that, Isolate Code no (Q4) sample was RHVD positive by RT-PCR although it was HA negative when tested by HA test. This agreed with Abd El-Moaty *et al.*, 2014 who said that haemagglutination test no longer a reliable test in diagnosis of RHDV and Guittre *et al.*, 1995 who mentioned that the application of RT-PCR assay was

more sensitive, ideal rapid and low cost tool for diagnosis and subtyping for RHVD.

From Table 5 and Fig. 4 revealed that phyelogenetic analysis of VP_{60} gene of RHDV strains in this study can be divided into two major clusters, the classic RHDV genotype5 and RHDV2. The alignment indicated that the RHDV\Vet-Alex-Q2, RHDV\Vet-Alex-Q7, RHDV\Vet-Alex-K11 clustered with the classic RHDV group, whereas the remaining RHDV\Vet-Abotaleb, RHDV\Vet-Alex-K15, RHDV\Vet-Alex-B18 and RHDV\Vet-Alex-B26 clustered with the RHDV2 group. The result revealed that either classic RHDV (G5) and the newly emerged RHDV2 were still circulated in rabbit populations in Egypt. Subsequently, the data obtained from flock sample history and phylogenetic analysis proved that all ages and breeds of rabbits become susceptible to that disease. This agreed with Le Gall-Reculé *et al.,* 2011 and Peacock *et al.,* 2017 who said that a virus, had a capsid protein sequence identity of about 80 per cent with RHDV2, was able to cause RHD in vaccinated and young rabbits (15–25 days old). While, Ewees. 2007) which proved that RHDV1 infect multiple breeds mainly adult age (more than 5 months) but rabbits (1-3months old) were less effected. Ruvoen-clouet *et al.,* (2000) said that adult rabbit more susceptible than young for infection by classic RHDV due to presence of ABH antigen in the epithelial of upper respiratory tract and digestive tract of adult in contrast young age rabbit. Also, collected samples from suspected cases exhibited vaccinated or not vaccinated rabbits are equal for infection which proved by (El-Bagoury *et al.*, 2014).

On comparing between the newly isolated RHVD strains, it was found from Table 6 that the nucleotide divergence within classical group varied from 0.9 – 4.5% while nucleotide variation among RHDV2 isolates ranged from 1.3-7.3% but there is maximum nucleotide difference between two groups about 14.4-20.6%. In the meanwhile, the new isolates also showed a maximum nucleotide divergence ranged from 11.2 to 21.2% when compared to commonly used vaccine strain (RHDV-Giza 2006). From these results, continuous distribution of original and appearance and gradual replacement of RHDV2 in Egypt may due to vaccine mismatch (Lopes *et al.,* 2015).

Current study revealed that there is amino acid substitution in S or A 476 E among the majority RHDV strains and vaccinal strain to newly emerged RHDV isolates. This change may cause changes with respect to polarity according to Esteves *et al.,* (2008) who identified positive selected codons (PSCs) in F region (amino acid number 476). They found that amino acid substitutions at the PSCs resulted in Changes in the polarity or charge of the protein that are important for the protein structure and protein–protein interaction.

The multiple alignment of deduced amino acid between the of classic RHDV isolates and commonly vaccinal strain(GIZA 2006) in comparison to RHDV2 showed 13 constant amino acid differences at position I 551 V,V 553 I ,k 562 T ,E 549 D ,T 548 S,T 542 A, I 526 L,T 518 N,T 487 A ,S 476 E ,V 473 I ,V 455 I , D 441 N. All these substitutions may be characterized as differential aa substitutions between them.

The average nucleotide identity between RHDV1 and RHDV2 ranged from 79.4-86%. This finding agree with other reports stating that nucleotide similarity between them is about 82.4% (Le Gall-Reculé. *et al*., 2013).

In-vitro replication system for RHDV is unachieved till now, so rabbit inoculation was the only way of isolating, propagating and titrating the infectivity of the RHDV (Metwally and Madboully, 2005; Em-bury-Hyatt *et al.,* 2012). The positive RT-PCR viral samples were inoculated into seronegative susceptible rabbits after wards the clinical signs, the mortality rate and post mortem were observed. In the present study mortality rate of isolates ranged from 20- 80% in accordance with Shakal *et al.,* (2011). From data exhibited from genetic analysis and pathogenicity, showed RHDV strains clustered into classic RHDV kill 60-90% from inoculated rabbits in contrast RHDV2 strains showed low virulence with average 20-40% mortality which agreed with (OIE.2018) & (Le Gall-Reculé. *et al*., 2013) who mentioned that RHDV2 less virulent than RHDV strains.

Conclusion: The present study revealed circulation of classic RHDV and newly emerged RHDV2 in affecting rabbit farm in Egypt causing high mortality rate and there is high genetic diversity between present isolates and vaccinal strain, so it is of necessity of preparation of vaccine as the available vaccine no longer provide protection against the disease.

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