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MOLECULAR IDENTIFICATION OF RHDV EGYPTIAN STRAINS BASED ON THE HIGHLY VARIABLE REGION OF VP60 GENE

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ABSTRACT

Rabbit Hemorrhagic Disease Virus (*RHDV*) is still considered highly contagious and fatal disease in Egypt although vaccination strategies. Until now there is no full detailed study concerning the antigenic evolution of *RHDV* in the Egyptian field. The circulating *RHDV* isolates in the different Egyptian governorates showed the existence of HA negative and positive as well as variable HA isolates without identification of these isolates on the molecular level. Sequence and phylogenetic analysis of the variable region (C-E) of VP60 gene for seven local isolates revealed the presence of both original (Giza97&Kal2011) *RHDV* as well as variant (KS2000, Kal2000, Kal2005, Giza2010 and Kal2012) are circulating in the Egyptian field till recent period with predominance of *RHDVa* subtype regardless to special period or location without evidence of replacement of *RHDVa* the original *RHDV*. Original *RHDV* strains showed completely different protean analysis from *RHDVa* strains as ten significant aa substitutions at region E amino acid have resulted in obvious changes in the antigenic index. Even Giza2006 vaccine strain showed unique amino acid substitutions from other Egyptian variant strains. In conclusion, the bioinformatics analysis may explain emergence of *RHDV* outbreaks although vaccination programs using locally isolated Giza2006 variant strain thatrequire to revise the vaccination strategies and extensive molecular epidemiological studies.

Keywords: RHDV, phylogenetic analysis, antigenic evolution

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1. INTRODUCTION

Rabbit hemorrhagic disease (RHD), first described in China in 1984 (Liu et al., 1984) causes hemorrhagic necrosis of the liver within three days after infection and with a mortality ratethat exceeds 90%. RHD has spread to large parts of the world and threatens the rabbit industry and related ecology.

Rabbit hemorrhagic disease virus (*RHDV*) is an icosahedral, non-enveloped, positive-sense, single-stranded RNA virus within the genus *Lagovirus*, family *Caliciviridae* (Meyers et al., 1991a and Ohlinger et al., 1990). RHDV virions are small sized between 35-40 nm of diameter (Valicek et al., 1990). The viral genome is composed of approximately 7.4 kb positive single-stranded RNA genome that encodes a 257 kD polyprotein and a subgenomic RNA of approximately 2.2 kb and both encoding for VP60 (Meyers et al., 1991b). There are two reading frames in the RHDV genome: a longer- ORF1 (7,034 nucleotides) that have post-translational processing at 8 proteolytic

cleavage sites. This polyprotein gives rise to several mature nonstructural proteins including a helicase, protease, and RNA-dependent RNA-polymerase, as well as the 60 kDa major capsid protein/antigen VP60 (p16, p23, p37, p30, VPg and VP60), and a shorter- ORF2 (353nucleotides), coding a VP12 protein and VP60 (Meyers et al., 1991a, Wirblich et al., 1996 and Meyers et al., 2000).The capsid protein VP60 consists of a shell (S) domain which is buried and comprises the N-terminal connected by a hinge to the protruding (P) domain that encompasses the C-terminal region and is exposed on the surface. The P domain can be subdivided into P1 (stem of arch) and P2 sub-domains (top of arch) (Neill,. 1992). Six distinct regions (A to F) can be distinguished in VP60; regions C and E are located in the exposed P2 sub-domain at the most exposed region of the capsid, displaying the greatest genetic variation (Neill,. 1992, Capucci et al., 1998, Schirrmeier et al., 1999). VP60 protein is an antigen recognized by the immune system of the rabbit host, and thus, plays an important role in the immune response against the infection with RHDV (Viaplana et al., 1997). Seven regions of high sequence variation were identified in P2 sub-domain that differs from other *caliciviruses*, dictates the binding specificities of histo-blood group antigens and interactions with rabbit tissue cells and elicits a significant immune response in rabbits and, hence, protect them from *RHDV* infection (Wang et al., 2013). Currently, the absence of a high-resolution model of any *lagovirus* impedes the understanding of its molecular interactions with hosts and successful design of an efficient anti-RHDV vaccine.

RHDV has only a single serotype (Capucci et al., 1995). Then the emergence of an antigenic variant strain or subtype of RHDV was identified as *RHDVa* (Capucci et al., 1998, Schirrmeier et al., 1999). It was noticed that *RHDVa* is replacing original strains of *RHDV* in Italy (Grazioli et al., 2000). Likewise, two recent French isolates belonging to the *RHDVa* antigenic subtype has been identified (Le Gall-Reculé et al., 2003). Also, the World Organization for Animal Health (OIE) has reported that the RHDVa subtype was responsible for the first ever recorded outbreak of RHD in Uruguay near the end of November of 2004.

The increasing number of published RHDV sequences on Genbank reflects the high interest in this virus, as sequence analysis allow tracing the genetic variability of the virus and performing phylogenetic analysis, providing information on the relationship of the strains and evolutionary dependencies.

In Egypt, RHDV is still representing a threat in the rabbit production farms in spite of vaccination programs. RHDV was first reported in Egypt in Sharkia Province, in1991 (Ghanem, and Ismail 1992). Many studies have been performed for diagnosis of the disease using different techniques either Hemagglutination test, Hemagglutination Inhibition test or using molecular tools as western immune blot and PCR technique (EI-Mongy 1998, Ibrahim et al., 1999, Salman 1999, Salman et al., 2008, Fahmy et al., 2010, Ahmed et al., 2011). Also, Giza2006 vaccine strain has been characterized as an RHDV variant by sequence analysis of full length VP60 gene (Ibrahim et al., 2012).

Until now there is no full detailed study concerning the antigenic revolution of RHDV in the Egyptian field, so the aim of this work is tracking the genetic revolution of RHDV in Egypt since 1997 and up till 2012 depending on sequence comparison

data of the main antigenic determinant regions (C and E) of VP60 gene between the different isolates representing the time intervals and to determine the situation and phylogenetic analysis of our local strains with the different classical and variant strains worldwide. Special approach concerning the existence of non heamagglutinating as well as RHDV with variable HA profile is needed in Egypt to discuss the genetic differences between the different RHDV phenotypic patterns for better vaccine design.

2. MATERIALS AND METHODS

2.1. RHDV Samples:

Seven local RHDV isolates were identified in previous work (in press) designated as Giza97, KS2000, Kal2000, Kal2005, Giza2010, Kal2011 and Kal2012 that were all positive for RHDV using RT/PCR technique performed with specially designed primers flanking C-E region of VP60 designed using Primer- Blast software to amplify a product length 600 bp :Upstream primer (6106-6125 bp):5' CCT GGA GGG TTT TCT ACG TG 3' and the downstream primer (6688- 6706 bp):5' AGA CGA CAG ACG CGA ACA T 3' that was synthesized by Bio Basic Inc. These isolates showed different HA patterns as Giza97, Giza2010 and Kal2011 were positive HA, while KS2000, Kal2000, Kal2005 showed variable HA profile but only Kal2012 was HA negative isolates.

2.2. Purification of highly variable region of VP60 gene

RT/PCR products of 600 bp obtained in previous work (in press) for each isolate were purified using agarose gel extraction kit Qiaquick (Qiagen, Germany).

2.3. Nucleotide sequence

Purified RT/PCR products representing 600 bp C-E region of highly variable region of VP60 gene were sequenced using BigDye® terminator v3.1 kit with the same primers used for RT/PCR in the automated sequencer ABI Prism 310 genetic analyzer at AGERI, Agricultural research center.

2.4. Sequence analysis:

Data were analyzed using Bioedit, MEGA 5, MegAlign software. The analysis was performed with other published RHDV strains that were selected to represent both genotypes original and variant RHDV strains and different geographical and temporal origin from Genbank. The accession numbers of used RHDVa strains are: Giza 2006(JQ995154), IN 05 USA (EU003578), NY 01 USA (EU003581), UT 01 USA (EU003582), Var Iowa 2000 USA (AF258618), Vt 97 Italy (EU250331), JX CHA 97 (DQ205345), WHNRH china (DQ280493), Hartmannsdorf (Y15425), 99-05 France (AJ302016), Triptis (Y15442), 00-Reu (AJ303106), Whn-1 (DQ069280), Pv 97 (EU250330), N11 Spain (JX133161) while original RHDV was represented by Spain AST 89 (Z24757), Rianham UK (AJ006019), Bs89 Italy (X87607), FRG 91 (M67473), KSA/ 96 (DQ189078), Bahrain 2006 (DO189077), Hagenow (Y15441), Frankfurt (Y15424), Czech V351(U54983), WX china 84, (AF402614), as well as RCV Italy (X96868) to be used as out group.

2.5. Phylogenetic analysis:

MEGA5 program was used for tree construction with Maximum likelihood method for constructing both nucleotide and deduced amino acid sequence phylogeny. Splits Tree program and Recombination detection program were used for detecting any recombination event.

2.6. *Protean analysis:*

Protean software from DNASTAR, Lasergene package, was used for prediction and annotation the structural character and topological features of a protein through

comparing surface probability plot, the antigenic index, hydrophilicity and flexibility plots.

3. RESULTS

3.1. Nucleotide sequence:

Sequence data were submitted to Genbank with accession numbers: Giza 97 (JX391954), KS2000 (JX409902), Kal2000 (JX429925), Kal2005 (JX436484), Giza2010 (KC920592), Kal2011 (KF646793) and Kal2012 (KC788211).

3.2. Sequence analysis

Multiple alignments of deduced amino acid sequences of highly variable region (C-E) of VP60 gene of local strains with other original and variant published strains performed with Bioedit software is shown at Fig (1).

3.3. Phylogenetic analysis

Phylogenetic tree of nucleotide sequence of RHDV highly variable region of VP60 gene at Fig (2) representing 11 original or classical RHDV strains, 12 variant RHDV strains (RHDVa) as well as rabbit calicivirus like viruses (RCV) and Spanish N11 as outgroup. This rooted tree is constructed by MEGA 5 software using Maximum Likelihood method with bootstrap values calculated from 1000 replicate. The variation of amino acid sequence according to the hemagglitonation activity of highly variable region C-E of VP60 are compared at Table (1) as Whn-1, Pv97, Frankfurt and Kal2012 are HA negative while Giza2010, Giza2006, Giz97 and Kal2011 are HA positive. Strains Hagenow, KS2000, Kal2000and Kal2005 has variable HA profile showing only the variable sites. The unique mutations were highlighted.

3.4. Protean analysis:

Surface probability plot according to Emini method for Egyptian strains was performed by protean analysis DNASTAR, Lasergene package is shown at Fig (3). Antigenic Index according to Jamesone-Wolf performed for Egyptian strains by Protean analysis DNASTAR, Lasergene package is shown at Fig (4).

Table (1): The variation of amino acid sequence of highly variable region C-E of VP60 according to the hemagglitonation activity

Strain/site	304	305	307	324	345	349	377	381	386	399	425	434	437
Whn-1	F	A	N	I	N	I	E	A	N	T	V	N	T
Pv97	Y	A	N	I	N	V	E	A	N	I	V	S	T
Frankfurt	Y	P	N	V	N	I	E	A	G	T	V	N	T
Kal2012	Y	S	N	I	D	I	E	A	N	T	I	N	T
Hagenow	Y	P	S	I	N	I	R	D	S	T	V	N	A
KS2000	Y	S	N	I	N	I	E	A	N	T	I	N	T
Kal2000	Y	S	N	I	N	I	E	A	N	T	I	N	T
Kal2005	Y	S	N	I	N	I	E	A	N	T	I	N	T
Giza2010	Y	S	N	I	N	I	E	A	N	T	I	N	T
Giza2006	Y	S	N	I	N	I	E	A	N	T	I	N	T
Giza97	Y	P	N	V	N	I	E	A	G	T	I	N	T
Kal2011	Y	P	N	V	N	I	E	A	G	T	I	N	T

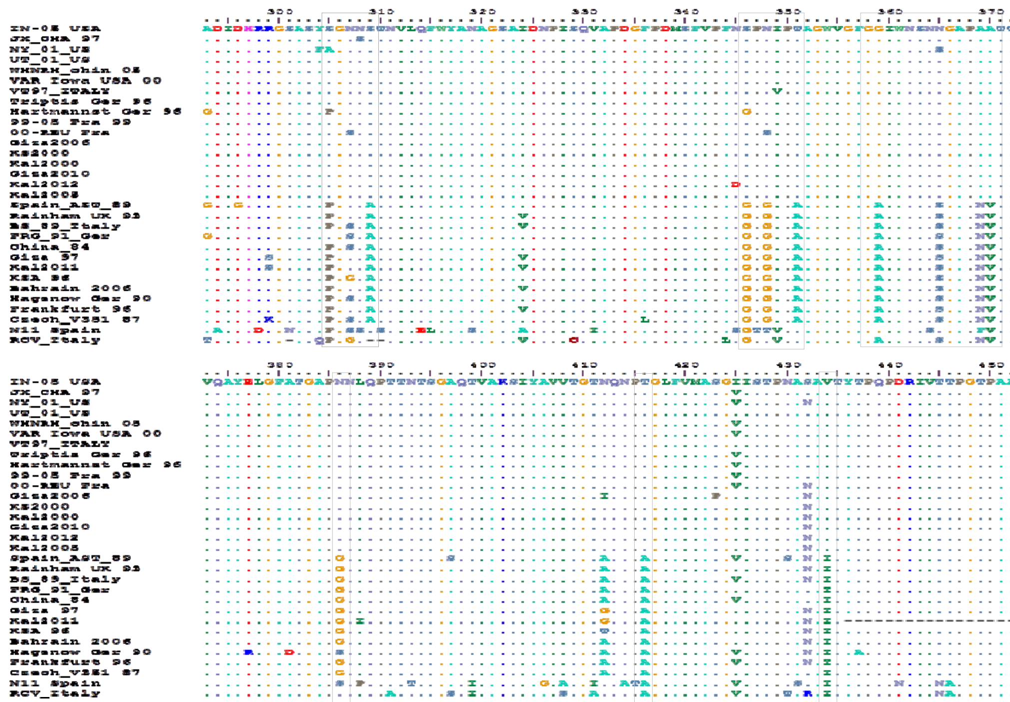

Fig (1): Multiple alignment of deduced amino acid sequences of highly variable region (C-E) of VP60 gene of local strains with other original and variant published strains.

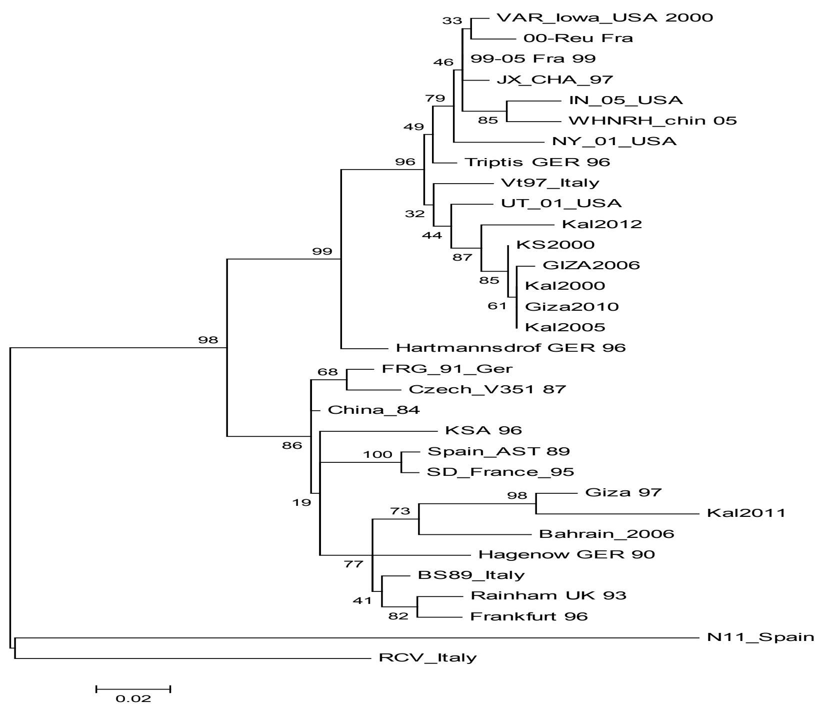

Fig (2): Phylogenetic tree of nucleotide sequence of RHDV highly variable region of VP60 gene representing 11 original or classical RHDV strains, 12 variant RHDV strains (RHDVa) as well as rabbit calicivirus like viruses (RCV) and Spanish N11 as outgroup.

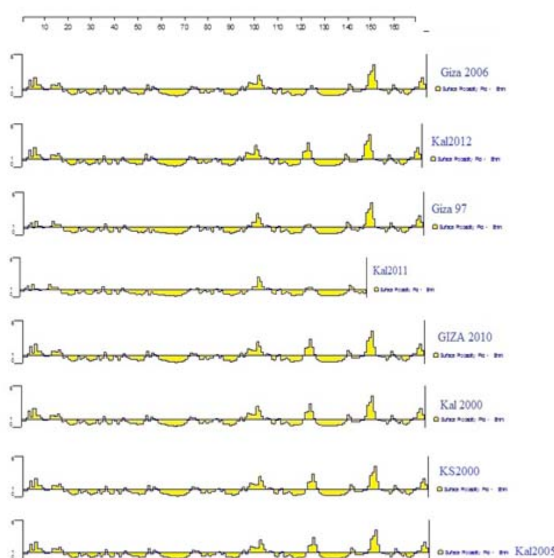

Fig (3) Surface probability plot according to Emini method for Egyptian strains by Protean analysis DNASTAR, Lasergene package

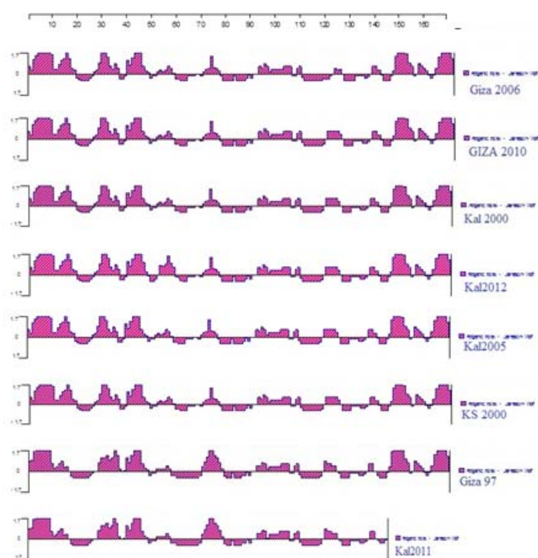

Fig (4) Antigenic Index according to Jamesone-Wolf performed for Egyptian strains by Protean analysis DNASTAR, Lasergene package

4. DISCUSSION

The molecular characterization of Egyptian isolates was performed using sequence analysis of highly variable regions (C and E) at P2 domain in the C-terminal part of VP60 that were amplified by RT/PCR technique.

The nucleotide sequences of the 7 Egyptian isolates collected from 1997 to 2012 were compared with RHDV published sequences on Genbank. The homology percentage results divided the 7 Egyptian isolates into 2 groups, Kal2000, Kal2005, Giza2010, KS2000 and Kal2012 showed high homology percentage with the variant Giza2006 vaccine strain as well as the American variant strains UT-01and IN-05 and other published variant strains, while the other 2 isolates Giza97 and Kal2011 showed high homology percent with original RHDV strains as Rainham and Bahrain.

The same results were obtained on the deduced amino acid sequence level that covered from 293-460 aa Fig (1). Egyptian isolates Kal2000, Kal2005, and Giza2010 showed 100% identity with each other on both nucleotide and amino acid sequence levels.KS2000 showed 99.8% homology with them on nucleotide sequence level but showed 100% homology with them on amino acid sequence level while Kal2012 showed 97.2% with them on the nucleotide sequence level and 99.3% on the amino acid sequence level.

The second group of Giza97 and Kal2011 isolates showed high homology 98.6 - 97.9 % with Rainham and Bahrain original RHDV in contrast with variant Giza2006 vaccine that showed 88-89.5% with them on amino acid sequence level and 84.9-88.1% on nucleotide sequence with the other Egyptian isolates.

These results give a conclusion that Giza97 and Kal2011 isolates can be defined as original RHDV strains while Kal2000,

KS2000, Kal2005, and Giza2010 isolates are defined as variant RHDV isolates.

The variability between variant Egyptian strains was very low 0 - 2.8% while it showed higher value between the original Egyptian strains 5.3% and up to 15% between the Egyptian variant and original strains. This is on the nucleotide sequence level, while on the amino acid level it showed lower variability 0 - 2.1% between variant Egyptian strains, 0.7% between original Egyptian strains and up to 11.9% between original and variant Egyptian isolates. These results agreed with the genetic diversity reported previously (Le Gall-Reculé et al., 2003, Oem et al., 2009).

For more clarification of the genetic relatedness of Egyptian isolates with the other published RHDV strains, phylogenetic analysis of nucleotide sequence Fig (2) showed two major branches or clusters, one representing the original RHDV subtypes and the second for RHDVa subtypes with a high bootstrapping value of (98), other than RCV and N11 as out group that are widely divergent from all other strains (79.5% and 76%) nucleotide identity respectively and also76.7% from each other.

Both phylogenetic trees (either nucleotide sequence based or deduced amino acid based) demonstrated that Egyptian isolates (Kal2000, KS2000, Kal2005, Giza2010 and Kal2012) are clustered with Giza2006 Egyptian variant strain previously identified within RHDVa clade (Ibrahim et al., 2012) but in a separate sub- genogroup with high bootstrapping value (87) from the other RHDVa strains. Kal2012 strain was divergent from other Egyptian variant strains in a separate branch within the sub-genogroup while KS2000 strain was partially divergent from others.

Egyptian isolates (Giza97 and Kal2011) are clustered together on a separate branch but divergent from each other, with high bootstrapping value (98) with known original RHDV strains in close relationship with Bahrain strain. It was noticed that nucleotide based tree showed that clustering of subgroups was more correlated with the year of isolation rather than the geographical location as previously reported (Le Gall-Reculé et al., 2003, Oem et al., 2009, Forrester et al., 2006 and Moss et al., 2002) except the branches of Egyptian strains either under original or variant clades were separated from others regardless the temporal or governorate of isolation.

The divergence between Giza97 and Kal2011 original RHDV strains at both phylogenetic trees indicated that non identical original RHDV strains were circulating in the Egyptian field for long time around 14 years with variation about 5.3% which is greater that the variation between the Egyptian variant strains isolated with long period gap as Kal2000 and Kal2012 that showed limited variation only 2.8%. Although the amino acid sequence variation between the two original strains was 0.7% , they showed different phenotypes as Kal2011showed 100% mortality only in young age rabbits less than 3 months in contrast to Giza97 that caused 100% mortality in adults only. It is not known whether a new original RHDV, closely related to Bahrain strain has been introduced to Egypt or these original strains were circulating harmlessly and re-emerged with this new geno and phenotype of Kal2011or they were responsible for vaccination failure as vaccination programs were performed using Giza2006 variant strain. These results were in contrast to the conclusion that probably gradually

replacement of original RHDV by the new variant and considered the increasing dominance of RHDVa is due to the selective pressure on the virus applied by vaccination (Oem et al., 2009).

So, phylogenetic analysis is the only reliable method for genotyping of RHDV strains either original or variant or even RCV and used to determine the genetic diversity and the genetic evolution of RHDV strains in each country as presented by many authors [10], (Le Gall-Reculé et al., 2003, Moss et al., 2002 and Matiz et al., 2006).

The multiple alignment of deduced amino acid Fig (1) of the two groups of RHDV original and variant showed two constant amino acid differences at region C positions P 305 S and A 309 S as well as ten significant aa substitutions at region E at positions G 346 S, G 348 N, A 351 T, A 359 G, S 365N, N 369 A, V 370 A, G 386 N, A 416 T and I 434 V. these positions were mentioned previously by many authors (Fitzner et al., 2012 and McIntosh et al., 2007). All showed that the most variant strains showed different amino acids mostly detected in region E spanning amino acid 344-434 of VP60. Also significant aa substitutions were identified between original RHDVs and RHDVa subtypes, two aa at region A (residues 37 & 50), two aa at region B (residues 207 & 219), two sites at region F (residues 476 & 480) and nine sites at E region (residues 346, 348, 359, 365, 369, 370, 386, 416 and 434). So, region E appeared to be hyper variable and preferential site for mutations (Oem et al., 2009).

The multiple alignment of nucleotide sequence (data not shown) revealed constant differences between the variant RHDV group and original RHDV that were corresponding to the same amino acid substitution at positions 913, 925, 1036, 1042, 1051, 1075, 1093, 1105, 1108, 1156, 1234, 1246 and 1300, so these positions could be considered as differential sites between original RHDV strain and variant ones and could be used as a marker between them.

The comparison between the two groups revealed the presence of some amino acid positions showing high tendency of substitutions at some strains at positions (307 at region C and 324, 412, 425, 432 at E region) fig (1).All Egyptian strains either original or variant showed aa substitution at position S 432 N. Three codons were reported previously (307, 432 and 476) that appeared to be under the positive selection (PSC). Amino acid substitutions at PSCs may impose changes in the polarity or the protein charge which is important for the protein structure or protein-protein interactions. The possibilities within PSC 432 from S or N to K aa could change the charge from neutral to positive but the amino acid possibilities within the other PSC 307, 476 could not change the neutral charge but showed polarity changes. It was suggested that changes at the 3 PSC can generate a putative N-glycosylation site which is known to allow the virus to increase the surface diversity and thus affect infectivity, protein folding, tropism, proteolytic processing and immune evasions (Esteves et al., 2008). Other findings showed that codon 137 at region E considered a strong evidence of positive selection proposing that the rising positive selection pressure observed throughout the history of RHDV is likely mediated by the host immune system as a

consequence of the genetic changes that rendered the virus virulent (Alda et al., 2010). Moreover five codons (307,412, 432, 476 and 572) within P1 subdomain were found to be subjected to strong positive selection as well as two codons 12 and 135 within the S domain that form the internal shell of the capsid so they are not directly exposed to immune recognition. These codons may influence antigenic diversity of the epitopes recognized by the host antibodies or by changing orientation or presentation of surface epitopes (Kinnear and Linde., 2010).

Other nucleotide positions showed high mutation rate in either original or variant strains (912, 972, 981, 1020, 1068, 1227, 1252, 1290, 1332 and 1426) but these mutations were without any change in the corresponding amino acids.

Meanwhile unique point mutations resulted in unique amino acid substitutions were recorded for Giza97 and Kal2011from other original RHDV strains at position A 897 T corresponding to unique amino acid substitution at R 299 S at region B as well as nucleotide mutation A 970 G corresponding to amino acid substitution at I 324 V at region C and nucleotide mutation C 1235 G corresponding to amino acid substitution at A 412 G at region E. On other hand, Kal2011 isolate showed unique nucleotide mutation C 1162 A resulted in amino acid corresponding to amino acid substitution at L 388 I at region E.

On the level of variant RHDV strains, Kal2012 showed unique nucleotide mutation A1033 G corresponding to amino acid substitution at N 345 D at region E while variant vaccine Giza2006 showed two nucleotide mutations at A 1235 T and T 1267 C corresponding to amino acid substitution at N 412 I and S 423 P respectively at region E.

Other unique nucleotide mutations were detected especially in Kal2011, Giza97, Kal2012, even the variant vaccine Giza2006 but all these mutations did not result in amino acid substitution.

The results showed that the amino acid substitutions were clustered mainly at region E of VP60 (73%), while region C contained 18% of aa substitutions and region D contained only 9% of them. It was noticed also these amino acid substitutions were grouped in the beginning of region E (44% of these substitutions at N-terminus 344-370) and the end (30% of these substitutions at C-terminus 412-434) of E region, while the central area of region E showed higher stability as it contain only one aa substitution agreeing with previous reported data (Capucci et al., 2008). These give evidence that the N-terminus of region E is responsible for differentiation between original and variant RHDV strains.

Special approach for detection of genetic differences based on the highly variable region of VP60 between heamagglutinating RHDV, non- heamagglutinating and HA variable profile was performed by multiple aligning of our Egyptian strains as well as published RHDV that represents the three HA characters table (1). The results showed that all amino acid substitutions were located at positions 305, 309, 324, 346, 348, 351, 359, 365, 369, 370, 386, 412, 416 and 434. All these substitutions were characterized as differential aa substitutions between the original and variant RHDV. The comparison between the non HA Pv97 strain with other HA RHDV strains revealed two aa

substitutions at position (S 305A), (T 399 I), (T 476 S) (Capucci et al., 2008), but our results showed that the aa substitutions of Pv97 strain were unique for it and was not common with any other HA negative strain.

Only one amino acid substitution was detected at position 425 between the 3 published HA negative strains (Whn-1, Pv97 and Frankfurt) as well as the variable HA strain (Hagenow) except the negative HA Egyptian variant Kal2012 and HA positive strains. Unfortunately, this aa substitution was found to be shared with HA positive Meiningen, Hartmannsdorf, Triptis and Eisenhüttenstadt German strains (Schirrmeier et al., 1999).

Even the phylogenetic analysis based on both nucleotide and amino acid sequences showed the distribution of heamagglutinating RHDV, non-heamagglutinating and HA variable profile according to their genetic type either original or variant as the phylogenetic trees were divided into 2 clades, the first clade included the variant China Whn-1 and Italian Pv97 with the variant Egyptian strains (Kal2005, Giza2010, Kal2000, Giza2006, KS2000 and Kal2012) in spite being HA positive, variable or negative. The second clade included the classical negative Frankfurt and variable Hagenow as well as Egyptian isolates Kal2011, Giza97 with positive HA. These results correlated with the non HA strain Whn-1that was clustered phylogenetically with RHDVa strains although not all of them were non HA or even with low HA capacity (Tian et al., 2007).

A similar results concluded that no common alteration at position 305, 399,476 in the HA negative strains but aa substitution at position T 399 I was recorded for polish GSK and ZD0 with variable HA profile and similarly to strain Pv97. Only amino acid change at A 506 S was found in BLA and Frankfurter strains as it may form a part of the discontinuous domain responsible for hemagglutination (Fitzner et al., 2012) as reported previously (Capucci et al., 2008).

Pseudoatomic model of RHDV revealed significant differences in the P2 sub-domain of the major capsid protein that may provide better explanation as it showed three putative pockets or cavities on the outer surface of the RHDV capsomer where one or more might contributed to HBGA binding (Wang et al., 2013). The surface of the P2 sub-domain was divided into seven regions of high variation V1 correspond to aa at positions (301-310), V2 (346- 351), V3 (362 – 370), V4 (384 – 388), V5 (411 -417), V6 (431 -435) and V7 (475-480). The interaction of V1-V5 regions or loops may give rise to different HBGA binding cavities C1, C2, C3 (Wang et al., 2012). Referring to multiple alignment based on amino acid table (1) we found that variant Whn-1strain had unique aa substitutions at Y 304 F and S 305 A falling on L1 loop, while variant Pv97 strain had unique aa substitution at I 349 V falling on L2 loop as well as at position T 399 I but outside the P2 sub-domain variable regions and position N 432 S falling on L6 loop but away from the putative cavities. Egyptian variant Kal2012 strain had unique aa substitution at N 345 D too adjacent to L2 loop. Also Hagenow strain with variable HA had unique aa substitutions at N 307 S falling on L1 loop, the other substitutions at E 377 R, A 381 D and T 437A outside the P2 sub-domain variable regions as well as position G 386 S falling on L4 loop. Frankfurt strain did not show any unique or common aa substitutions with other HA negative strains within variable region C-E

but Frankfurt was reported previously to share two aa substitutions within region F at N 481S with HA negative polish strains (BLA and OPO) falling too adjacent to L7 loop as well as at position A 506 S falling outside the P2 sub-domain variable regions and both two aa substitutions were away from the putative cavities (Fitzner et al., 2012).

These results leading us to a conclusion that there may be other locations contribute to HBGA binding affinity outside the variable region of VP60 or there are other factors affecting the HA activity or variable HA profile as the different interactions between capsomers, domains and sub-domains depending on previous findings that RHDV capsomers has a hat shaped prominence at the P2 region, implying a specific antigen structure and antibody binding site (Hu et al., 2010). In addition, the RHDV CC capsomer interact with 2 adjacent AB capsomers via P2 subdomain and no interactions were observed between CC capsomers and 2 other adjacent AB capsomers. Inside AB capsomer, both P1 and P2 subdomains are involved in interactions, while only P2 subdomains are involved for CC capsomers. Also the pseudo atomic models of RHDV VP60 based on EM map revealed the rotation of RHDV VP60 P domain with respect to its S domain (Hu et al., 2010).

No recombination events could be detected in nucleotide sequences of Egyptian strains when Egyptian strains were examined by Splits tree program Fig (6) or recombination detection program. Only Hartmannstdorf strain showed intermediate branch between original and variant RHDV strains by Splits tree program and this is supported by the aa multiple alignment as this variant strain shared the specific aa substitutions at positions S 305 P and S 346 G with the original RHDVs agreeing with previous findings(Abrantes et al., 2008).

To study the effect of these nucleotide mutations and amino acid substitution on the antigenicity of Egyptian strains and to evaluate the emergence of outbreaks although vaccination programs using locally prepared vaccine (Giza2006) and causes of vaccination failure, Protean software was used for prediction and annotation the structural character and topological features of a protein through comparing surface probability plot fig (3),the antigenic index fig (4),as well as hydrophilicity and flexibility plots .

Surface probability peaks correlate well with exposed protein regions, but the absolute magnitude of these peaks is not always meaningful (Jameson and Wolf., 1988). The comparison between the original and variant Egyptian isolates showed 10 peaks expressing the exposed regions of this protein. The most prominent peaks were corresponding to positions (294-304) located at regions B and C, (306-311) at C region, while (345- 350) and (363- 370) representing at the N terminus of region E, (386- 396) in the middle of region E, in addition to (412- 416) and (436- 444) at the C-terminus of region E. The major difference was at peaks (345- 350) and (363- 370) representing at the N terminus of region E as these peaks were completely absent in the original (Giza97 & Kal2011) strains in contrast with other Egyptian variants. Also the peak (386- 396) in the middle of region E was narrower in original (Giza97 & Kal2011) strains than other Egyptian variants. Moreover original (Giza97 & Kal2011) strains showed smaller magnitude peaks at (412- 416) than other

Egyptian variants with strange exception of variant Giza2006 local vaccine that showed very diminished peak. The other peak (436-444) of C-terminus of region E showed no difference between the original or variant.

Original RHDV Giza97 and Kal2011 strains showed different peak shape from all other Egyptian isolates at the area 1-28 aa of the antigenic index plot Fig (4) and surface probability plot Fig (3) corresponding to 294- 321 aa of VP60, this area showed unique amino acid mutation for them from other published RHDV strains (R 299 S) falling in region B of VP60, as well as amino acid substitutions that differentiate original RHDV strains from variants laying in this area (A 305 P and S 309 A at region C of P2 subdomain). These mutations also affected the hydrophilicity plot of Giza97 and Kal2011 strains but without any effect on the flexibility of this region. These aa substitutions were located at the loop L1 (301- 310) which was identified previously as contiguous stretch of mostly hydrophilic residues, most exposed loop on the surface of the RHDV capsomer that lies juxtaposed to three putative HBGA binding pockets, hypothesized to be a primary determinant of RHDV host interaction and effective epitope in RHDV as it plays a critical role in defining RHDV antigenicity (Wang et al., 2013). So these mutations between original and variant strains may be sufficient for reducing the positive effects of vaccination when heterologous vaccines are used.

Giza97 and Kal2011 strains showed different peak shape of the antigenic index plot from other Egyptian isolates at the area 346- 363 aa (53-70) due to the 4 differential aa substitutions between original and variant RHDVs (S 346 G, N 348 G, T 351 A and G 359 A at region E).Also areas 364-381 aa (71-88) and 385-390 aa (92-97) showed larger peak shape were due to 4 differential aa substitutions between original and variant RHDVs (N 365 S, A 369 N, A 370 V and N 386 G at region E) as well as a unique aa substitution for original RHDV Kal2011 strain (L 388 I at region E). These substitutions affected the surface probability of Giza97 and Kal2011strains as well as the hydrophilicity plot was more hydrophobic at the same area. Also the flexibility plot showed a change specific for Giza97 and Kal2011 strains at the same area of variation.

The antigenic index plot showed a different peak shape of Giza97 and Kal2011 isolates in at the area 28- 40 aa that was due to aa substitution (I 324 V at region C) as in some of the original RHDV strains and another unique peak shape of variant Kal2012 strain at position 45- 64 due to the unique aa substitution of Kal2012 (N 345 D at region E) but these mutations did not result in any change in the surface probability, hydrophilicity or flexibility plots.

Antigenic index showed different peak shape of Giza97 and Kal2011 original RHDV strains and a smaller peak of variant Giza2006 vaccine strain at area 409-421 aa (116-128) and area 424-442 aa (131-148). These areas included a differential aa substitutions between original and variant RHDVs (T 416 A and V 434 I at region E) and aa substitution in some original RHDV strains (S 432 N at region E) for Giza97 and Kal2011as well as unique aa substitution for Giza97 and Kal2011 (A 412 G at region E) from other original RHDVs. The antigenic index change of Giza2006 was due to two unique aa substitution (N 412 I and G 424 P at region E) from other compared RHDV strains either original or variant. These aa substitutions has affected the surface

probability and hydrophilic pattern for these strains at corresponding area. From all these protean analysis it is clear that the original RHDV Egyptian strains showed different topology in all protean analysis parameters from other Egyptian variants.

These amino acid substitutions were reported previously, as 5 differential amino acid within position 281-321 and 359-386 residues resulted in diversity of hydrophilicity plot, flexible regions, antigenic index, surface probability plot suggesting that these residues are located in external region of VP60 protein (P2 region).while 6 aa substitutions at position 11-280 has resulted in diversification of hydrophilicity plot, flexible regions, antigenic index except surface probability plot, suggesting that these residues locate in internal region of VP60 protein (Tian et al., 2007).So other studies have used the antigenic index for evaluating the vaccine efficacy as the similar antigenic indexes showed full protection for each other while the divergent antigenic index showed approximately 50% protection (Wang et al., 2012).

This may lead us to conclude that Giza2006 vaccine that showed different antigenic index and surface probability profiles from other local variant strains and circulation of both original and variant RHDV strains may be responsible for emerging of new outbreaks in spite of vaccination with this local strain.

Regarding to the appearance of RHDV recently in young age rather than typical old age previously reported in Egypt Kal2011 (El-Nahas., 2011) and our recent strain Kal2012, we tried to trace this phenomenon on the molecular level, but unfortunately no common antigenic features or amino acid substitutions were detected between them. As Kal2011 is original RHDV positive HA while Kal2012 is variant RHDV of negative RHDV. The only recorded strain affecting the young age rabbits was the Spanish N11strain but our results showed wide divergence between (Kal2011 and Kal2012) and the Spanish N11 strain either by phylogenetic analysis or by nucleotide or amino acid sequence multiple alignment. So, more analysis will be required for both these strains by full genome sequencing and also more epidemiological studies are needed for more clarification of this issue.

In conclusion, this small scale analysis covering from 1997-2012 locally isolated RHDV showed that both original and variant RHDV are circulating in the Egyptian field till recent period with predominance of RHDVa subtype in the Egyptian field regardless to special period or location without evidence of replacement of RHDVa the original RHDV.

Original RHDV strains showed completely different protean analysis from RHDVa strains that may give an indicator for the efficacy of cross protection obtained due to vaccination with variant based vaccine. The results showed that all amino acid substitutions clustered within region E of VP60have resulted in obvious changes in the antigenic index. Amino acid substitutions at positions 324, 345, 424 and 432 did not affect any protean analysis except the antigenic index. The flexibility plot showed the minimal changes as it was affected only by amino acid substitutions at positions (346, 348, 351, 365, 359 and 370).

Although the original RHDV Giza97 and Kal2011 Egyptian strains showed 94.7% nucleotide identity between them, they showed 99.3% homology on amino acid

level and showed nearly identical protean profiles.

Also Kal2012 variant RHDV strain showed slightly lower identity percentage with other variant Egyptian RHDV strains either on nucleotide or amino acid levels but it showed only a changed antigenic index from other variant Egyptian RHDV strains without affecting the other protean analysis, while Giza2006 variant vaccine showed high homology 99.5- 96.7% with other variant Egyptian RHDV strains but it showed clearly different antigenic index, surface probability plot and hydrophilicity plot without affecting the flexibility plot due to the two unique amino acid substitutions at 412 and 424 of region E at VP60.

This leading to revise the vaccination strategies depending on this vaccine, as we recommend to prepare bivalent RHDV vaccine including both original and variant RHDVs to withstand the affection with either types and to change Giza2006 strain with other Egyptian variant strain that showed identical antigenic index with other Egyptian variants.

Conclusion

Continuous monitoring and molecular analysis of the RHDV strains present in Egypt should be performed. Moreover, both new and established vaccines should be evaluated through challenging with both original and RHDVa subtypes.

Also complete genome sequences of Egyptian RHDV strains are needed to allow identification of changes in virus sequences that may interpret the change in virus phenotype.

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التعريف الجزيئي للعترات المصرية بناءا على المنطقة عالية التباين في الجين الخاص ببروتين 60.

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الملخص العربي

لايزال مرض النزف الأرنبي الفيروسي واسع الأنتشار والأعلى في الوفيات في مجال تربية الأرانب في مصر على الرغم من برامج التحصين وإلى الأن لا يوجد دراسة مفصلة عن التطور الأنتيجيني لفيروس النزف الأرنبي في المزارع المصرية. وقد أظهرت الدراسات السابقة ان عترات فيروس النزف الأرنبي المنتشرة في المحافظات المصرية المختلفة سالبة وموجبة ومتغيرة لإختبار التلازن الدموي ولكن بدون توصيف جزيئي لهذه العترات. نتائج تحليل التتابع النيوكليوتيدي و الفايلوجيني للمنطقة عالية التباين (C–E) للجين الخاص ببروتين 60 في هذه الدراسة لعدد سبعة من المعزولات المحلية أظهرت وجود فيروس النزف الأرنبي بنوعيه الفرعيين; النوع الأصلي متمثل في العترات (جيزة 97 و القليوبية 2011) والنوع الثاني المتباين (كفر الشيخ2000 ، قليوبية 2000 ، قليوبية 2005 ،جيزة2010 و قليوبية 2012) منتشرين في الحقول المصرية مع تفشي الفيروس من النوع المتباين بدون ارتباط بفترة زمنية أو موقع محدد وبدون دليل على حدوث احلال عترات من النوع المتباين للعترات الأصلية. كذلك فإن العترات الأصلية للفيروس أظهرت إختلاف كامل عن العترات من النوع المتباين عن طريق تحليل Protean بسبب عشرة اختلافات مميزة على مستوى تتابع الأحماض الأمينية في المنطقة (E) ادت إلى اختلافات واضحة في الدليل الأنتيجيني. حتى العترة جيزة2006 من النوع المتباين المستخدمة لإنتاج التحصين محليا أظهرت وجود طفرتين فريدتين عن جميع العترات المصرية الأخرى حتى من النوع المتباين. ومن هنا يمكن استنتاج ان استخدام طرق Bioinformatics يمكنها ان تشرح او تفسر ظهور أوبئة لفيروس النزف الأرنبي على الرغم من برامج التحصين باستخدام عترة جيزة2006 المحلية الأمر الذي يتطلب مراجعة برامج التحصين والتوسع في استخدام الدراسات الوبائية على المستوى الجزيئي.

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