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Molecular Characterization of a Haemagglutinating and Non Haemagglutinating Rabbit Haemorrhagic Disease Viruses From Egypt

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Key words

Rabbit Hemorrhagic Disease Virus, haemagglutinating, Non-haemagglutinating, VP60 gene, Sequence analysis

ABSTRACT:

Rabbit viral haemorrhagic disease (RVHD) is a highly contagious, peracute and acute viral disease of both wild and domestic rabbits caused by rabbit haemorrhagic disease virus (RHDV). RHDV is considered as a haemagglutinating virus; however, there is a growing evidence for presence of non haemagglutinating RHDV strains. In this study, two RHDVs have been characterized. The two viruses were selected from ten different infected rabbit flocks. One of them had haemagglutination (HA) activity and the other one lacked HA activity. HA test was carried out on both isolates at 4°C using human red blood cells (RBC) type 'O'. RT-PCR was performed on the liver samples of the ten infected rabbit flocks using specific primers targeting VP60 gene. The obtained partial VP60 gene sequences of the two selected strains were aligned with other haemagglutinating and non haemagglutinating strains of RHDV in the world, including ''RHDVa'' strains as France 99-05, France-Reu-00, Germany-Triptis and China TP. Phylogenetic analysis showed that the two strains were genetically different. Haemagglutinating strain was similar to Rainham strain which is low haemagglutinating virus while non haemagglutinating strain was similar to whn-1 strain which is non haeagglutinating virus. The study confirmed presence of non haemagglutinating RHDV in rabbits reared in Egypt and suggested that VP60 might be related to HA activity.

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1. INTRODUCTION

Rabbit haemorrhagic disease (RHD) is a highly contagious, highly fatal, peracute and acute viral disease of both wild and domestic rabbits caused by rabbit haemorrhagic disease virus (RHDV). The disease was characterized by haemorrhagic lesions affecting, in particular, the liver and lungs, with high morbidity and mortality rates. At present, the disease has spread all over the continents (Tian et al., 2007). RHDV belongs the family *Caliciviridae*, genus *Lagovirus*, (Van Regenmortel et al., 2000) that also includes European brown hare syndrome virus (EBHSV) (OIE, 2010). RHDV genome is a linear single stranded RNA of 7437 nucleotides (Meyers et al., 1991). The genome comprises two open reading frames; the longer one (7034) encodes non-structural viral proteins VP16, VP23, VP37, VP30, virus protein genome linked (VPg), and a structural capsid protein (VP60), and the shorter one (353 nucleotides) encodes for viral protein VP12 with so far unidentified functions (Myers et al., 1991). Capsid protein VP60, the main structural protein of RHDV, plays a very important role in diagnosing virus, inducing protective immunoreaction against RHDV, and is exploited for vaccine generation (Tian et al., 2007).

Only a single serotype of RHDV is known to exist (Capucci et al., 1995). However, an antigenic variant strain or subtype known as RHDVa has emerged. The spread of RHDVa in different geographical locations suggest a selective advantage for infectivity or replication of RHDVa over the original serotype of RHDV (McIntosh et al., 2007). Most of the RHDV strains have haemagglutination activity. However, a few RHDV isolates displayed different haemagglutination characteristic under standard conditions contrasting to conventional RHDV strains, e.g., RHDV Blaszki and Whn-1 strains gave negative results in the haemagglutination

test (Kesy et al., 1996), and RHDV Rainham strain failed to haemagglutinate in standard conditions at normal temperature but haemagglutinated at 4 °C (Capucci et al., 1996). All these strains including RHDVa strains were indistinguishable in epidemiology, clinical symptoms, pathological lesions, ELISA assay from normal isolates of RHDV (Tian et al., 2007).

Here we report identification and capsid protein VP60 gene sequence analysis of a haemagglutinating and non-haemagglutinating RHDVs from Egypt.

2. MATERIALS AND METHODS:

2.1. Samples:

Ten field samples of rabbit livers were collected from freshly dead domestic rabbits at field of Minoufiya governorate, Egypt. The age of dead rabbits was above two months. The rabbits were of New Zealand breed. Prior to death, the rabbits displayed signs of low spirit, urgent breath, yelping and struggling motion. Some rabbits hemorrhaged from nostrils. Parenchymatous organs, in particular, livers and lungs presented haemorrhagic lesions.

2.2. Sample preparation:

A total of 1 g of liver tissue of every freshly dead rabbit was homogenized in 4 ml of phosphate-buffered saline (PBS; PH 7.2) and then repeated freeze thawed three times, and centrifuged at 4000 rpm for 30 min. The liver extract obtained from each rabbit was used for virus identification or stored at 70 °C.

2.3. Quantitative plate haemagglutination test (pHAT):

The liver extracts from the ten field samples were tested for HA activity and titre in micro HA plates according to (OIE, 2010, Shakal et al., 2011). Briefly, duplicate sealed round-bottom micro-titre plates containing two-fold dilutions of each liver extract were incubated with an equal volume of washed 0.75% human RBCs type "O" at 4 °C. Lattice and button shapes were recorded for each sample after one hour. The reciprocal of the end dilution (last well giving complete HA) was considered the end titre. Results were considered positive when having an agglutination end-point dilution of > 1/160 (OIE, 2010, Shakal et al., 2011).

2.4. One-step RT-PCR:

Viral RNAs were extracted from the samples of liver suspension with RNeasy (QIAGEN, Germany). Five microliters of purified RNA extracts from the livers of infected rabbits were used in 25 µl reaction mixtures using the Thermo Scientific Verso One-Step RT-PCR Kit (ABgene ® UK). RHDV upstream and downstream specific primers were used according to (Vende et al., 1995). The used primers were P33: 5'- CCACCACCAACACTTCAGGT -3' (6473–6492) and P34: 5' CAGGTTGAACACGAGTGTGC-3' (6992–7011), and the target region was 538 bp.

The reactions were heated at 50°C for 15 min followed by verso inactivation at 95°C for 2 min then 35 cycles of 95°C for 1 min, 56°C for 1 min, 72°C for 2 min, followed by 72°C for 10 min. PCR products were electrophoresed on a 1% agarose gel in 1X Tris–acetate–EDTA (TAE) buffer (40 mM of Tris and 2 mM of EDTA, with a pH value of 8.0) containing of ethidium bromide for 45 min at 100 V, then visualized and photographed under 304 nm UV light (UV Transilluminator**,** Major Science).

2.5. Nucleotide sequence analysis:

PCR products sequencing were carried out in Animal Health Research Institute. Sequencing was performed in both directions with virus specific primers. Sequences were analyzed using BioEdit program (Hall 1997). This program was also used to read the sequencing electropherograms to exclude nucleotide ambiguity. The phylogenetic analysis was based on the deduced amino acids of 453 nucleotides of VP60. The detected sequences were compared with others deposited in GenBank by multiple alignment with the Clustal W included in Bioedit software. Phylogenetic relationships were evaluated by the Neighbor Joining method present in the MEGA version 6 software (Tamura et al., 2013) with 1000 bootstrap replications.

3. RESULTS:

3.1. HA activity:

Liver extracts prepared from freshly dead rabbits showed variable HA titres ranging from 0 to 2^{14}.Samples number 1, 3,5, 7, and 9 were HA positive. The titer range was from 2^{11} to 2^{14}. Sample 1 HA titre was 2^{14}. Samples number 2, 4, 6,8,10 were HA negative.

3.2. One-step RT-PCR:

The RT-PCR results showed that all samples either HA positive or negative were all positive for RHDV. The amplified PCR products visualized by UV in agarose gel electrophoresis revealed size of 538 bp (Fig. 1).

3.3. Phylogenetic and sequence analysis:

Phylogenetic analysis of partial deduced amino acid sequences of VP60 gene showed that RHDV of sample number 1 and sample number 4 were genetically different. They were located in two different clusters. RHDV of sample 1 was close to low haemagglutinating Rainham strain and RHDV of sample 4 was close to non haemagglutinating strains Whn-1 and Giza 2006 (Fig.2).

Comparison of amino acid sequence of the two RHDV strains with other RHDV strains of different HA activity showed that RHDV of sample1 similar to Rainham strain and RHDV sample 4 is similar to Whn-1 strain (Table 1).

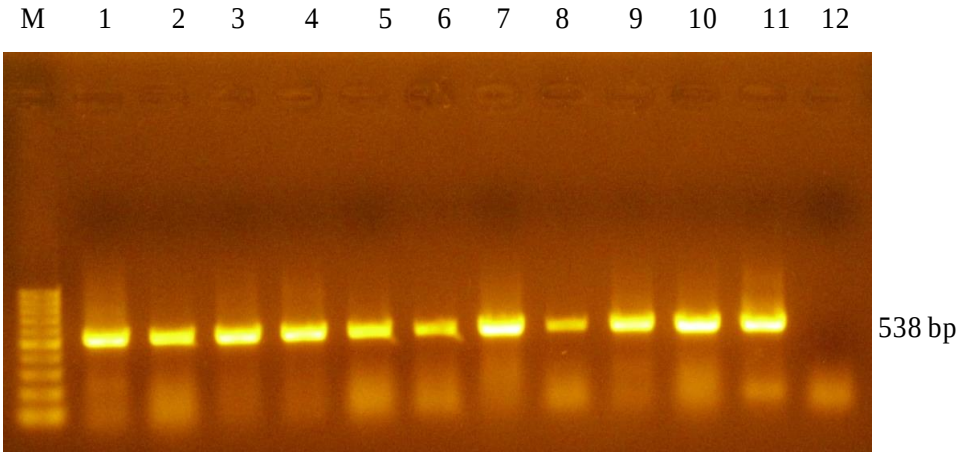

Fig.1: RT-PCR products of RHDV infected samples 1-10. Samples number 1,3,5,7 and 9 were haemagglutinating viruses. Samples number 2,4,6,8 and 10 are non haemagglutinating viruses. Well number 11 is positive control. Well number 12 is negative control. Expected size is 538bp.

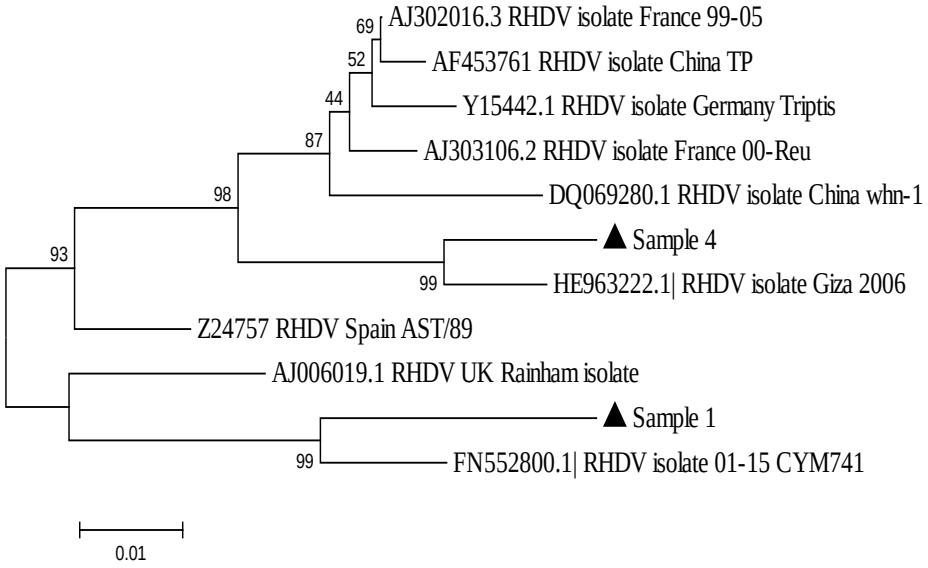

Fig.2: Phylogenetic analysis of partial VP60 protein sequences of different RHDV isolates.

Table 1: variation of amino acid sequences of VP60 gene of different RHDV isolates

	Amino acid site						
Strain	425	430	432	434	468	476	480
Sample1	I	N	N	I	R	S	T
Rainham	V	N	N	I	R	S	A
Sample 4	I	N	N	V	R	T	S
Whn-1	I	N	N	V	K	A	T
France 99-05	V	N	S	V	R	A	T
AST/89	V	S	N	I	R	T	A

4. DISCUSSION:

Although there is only a single serotype of RHDV (Capucci et al 1995), RHDV strains can be divided into two major clusters, the original RHDV subtypes and the RHDVa subtypes. The first antigenic variant of RHDV was detected by Capucci et al., (1998) and they designated as RHDVa (Capucci et al., 1998). All these strains including RHDVa strains were identical in epidemiology, clinical symptoms, pathological lesions, and ELISA assay (Tian et al., 2007).

Haemagglutination test using human red blood cells (RBC) type "O" is the primary test for routine field and laboratory diagnosis of RHDV (Capucci et al., 1991). However, new types of virus particles with different haemagglutinating properties have been reported. In Egypt, previous studies (Shakal et al., 2011, Abd El-Moaty et al., 2014) have reported detection of non haemagglutinating RHDV strains. In the current study, we analyzed a partial nucleotide sequence (representing residues from 425 to 480) of VP60 gene of both a haemagglutinating and a non haemagglutinating RHDV strains searching for any potential genomic difference. The highest variability within the complete VP60 was observed between amino acids 340 and 440 according to previous reports (Capucci et al., 1998, Schirrmeier et al., 1999).

By the comparison of partial VP60 deduced amino acid sequences of our strains, non haemagglutinating whn-1 strain, low haemagglutinating Rainham strain, conventional AST/89 strain and ''RHDVa'' France- 99-5 strain, the six strains have no significant deviation in amino acid composition (Table 1). The amino acid comparison of the two RHDVs of the current study with other RHDV strains showed that sample 1 had high similarity with the low haemagglutinating Rainham strain (only haemagglutinate at 4 °C). Amino acid sequences of sample 1 were identical with Rainham strain at positions 430, 432, 434, 468,476. Amino acid sequence of sample 4 was more similar to non haemagglutinating Chinese strain whn-1. The amino acids were identical at positions 425, 430, 432, 434. At position 468 sample 4 had R and Whn-1 strain had K. Therefore, the difference in amino acid sequences in these positions of VP60 gene among RHDV strains with different HA activity might play an important role in the haemagglutination activity. However, it would be necessary to demonstrate by further experimental studies what residues in VP60 protein or even in other proteins take critical part in the haemagglutination activity.

The presence of non haemagglutinating RHDV is not completely understood. Many hypotheses attempted to explain the relatedness of non haemagglutinating RHDV to classical RHDV. These hypotheses were fully discussed by (Abd El-Moaty et al 2014). It was reported that the loss of haemagglutinating activity of RHDV is associated with the chronic stage of RVHD (Granzow et al., 1996). Also it was reported that lack of HA activity due to the RHDV core like particles (CLP) or smooth particles or s-RHDV (Capucci et al., 1991). Abd El-Moaty et al., (2014) concluded that there is no satisfactory theory explaining the variable or negative HA profiles as they have no clear origin or specific mechanism. Furthermore, they suggested that HA negative isolates may recognize other cell receptor at the rabbits other than that recognized at the human RBCs type O.

It could be concluded that partial sequences of VP60 gene of two RHDVs from Egyptian field were identified. One of them haemagglutinated human RBC
type O and the other one did not haemagglutinte human RBC at 4° C. The results of the current study confirmed the finding of previous Egyptian studies regarding

circulating of non haemagglutinating RHDV strains in rabbits reared in Egyptian field. The study also confirmed that HA test is not reliable in diagnosis or typing of RHDV field isolates. Also, the study suggested the HA activity of RHDV could be related some residues in VP60 protein.

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