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Molecular detection of viral diarrhea in rabbits

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ABSTRACT

Enteric diseases represent a major problem in rabbit production. These causes high losses as result of mortalities and decreased conversion indexes. This study focused on viral agents (e.g., Astrovirus, Coronavirus (CoV), Rotavirus (RV) and Rabbit Hemorrhagic Disease Virus (RHDV)) as one of the causes of diarrhea in rabbits. 140 samples were collected from commercial farms located at Menofia and Qalyubia governorates, Egypt, during the period from October 2020 to May 2021. Our study focused on live and dead rabbits who showed diarrhea at the age of 7 days and up to 150 days of age. The most apparent symptoms were diarrhea, and the postmortem examination showed the presence of localized area of necrosis in different organs and splenomegaly. All samples in the current research came up negative for Astrovirus, Corona virus and Rota virus using Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) and though some samples were positive for RHDV in the liver and intestinal samples of dead rabbits with enterocolitis. Sequencing and phylogenetic analysis of the VP60 gene of the RHDV strain showed that it is related to RHDV2. Genetic analysis of our strain (RHV-Egypt-BenhaF954-2022) was clustered with G1.2/RHDV2/b strains with homology 97.1%. Homology with Egyptian classical strains were 96.6% with G1.1C/RHDV and 87.6% with G1.1a/RHDVa. The genetic diversity between the study obtained strain and other reference RHDV strain is indicative to probability of antigenic diversity.

1. INTRODUCTION

Intestinal infections in rabbits are a common cause of diarrhea, which can quickly lead to serious complications. On commercial meat farms, enteritis outbreaks are prevalent, resulting in low rabbit welfare, high rabbit mortality, and severe economic losses for rabbit growers (Xie et al., 2017).

Rabbit enteritis or rabbit enterocolitis (REC) is an intestinal disease characterized by various symptoms. Many viruses e.g., rotavirus, coronavirus, parvovirus, adenovirus, and caliciviruses, have been isolated from infected rabbits. Also, Astrovirus lead to gastroenteritis in most animal species and humans (Cerioli and Lavazza, 2006).

Lapine Rotavirus (LRV) cause acute viral gastroenteritis in rabbits it considered milder pathogen (Thouless et al., 1988), because it is associated with enteric disease in post-weaned rabbits and may be part of the etiology of more severe enteritis outbreaks that result in "enteritis complex" due to the presence of other viruses, bacteria (E. Coli, Clostridium spp.), and parasites. The rabbit coronavirus, also known as RbCoV, belongs to the family Coronaviridae. In the small intestine, the virus replicates, leading to necrosis of apical villi and, eventually, diarrhea (Descoteaux and Lussier, 1990).

Domestic and wild rabbits both are susceptible to the lethal viral disease known as rabbit viral hemorrhagic disease (RVHD) (Ferreira et al., 2006). Internal organs such as the liver, trachea, and lungs might have petechial hemorrhages (OIE, 2010) because of virus-induced hyper-coagulopathy. Young rabbits, aged from 4 to 8 weeks, or soon after being weaned, were found to be infected with the virus (Teifke et al., 2002). The virus occurs as local, sporadic outbreaks in young rabbits (Ji et al., 1994).

It first appeared in Egypt in 1991 in the governorate of Sharkia, and then spread all over Egyptian provinces (Ghanem and Ismail, 1992; Hemida et al., 2020). It is transmitted via oral, nasal, conjunctival, and vector-like insect routes (Urakova et al., 2019).

The causal agent (Rabbit hemorrhagic disease virus - RHDV) belongs to the family *Calicivirida, genus Lagovirus* (Parra and Prieto, 1990), A virus has a 7.4 kb single-stranded RNA genome that is positive-sense and non-enveloped. Belong to the Caliciviridae family, genus Lagovirus (Abrantes et al., 2012).

This virus is subdivided into two genogroups: RHDV (GI) and European Brown Hare Syndrome Virus (EBHSV) (GII). Each genogroup is divided into many strains (Le Pendu et al., 2017). The combination of classical and variant viruses increases the diversity of this virus species (Lopes et al., 2018). Because they both have two open reading frames, RHDV and RHDV2 have identical genomic structures (ORFs). ORF2 considers VP10, a minor structural protein, while ORF1 considers RNA-dependent RNA polymerase and the major capsid protein (VP60) (Dalton et al., 2015).

The most immunogenic protein, VP60, is the RHDV capsid's primary structural protein (Awad and Kotb, 2018). The

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VP60 gene of the virus was chosen as a target for the RT-PCR assay, a common method of general rabbit screening (Le Gall-Reculé *et al.,* 2017).

The aim of the current study was to identify the viral causes of diarrhea in rabbits by PCR techniques –focusing on the relatedness of our isolates and the other classic and variant isolates which are currently present in gen bank.

2. MATERIAL AND METHODS

Ethics statement

Following national approval of the protocols by the Research Ethics Board of the Faculty of Veterinary Medicine at Benha University, the procedure was carried out in accordance with the recommendations of the animal welfare committee (No.: BUFVTM 22-10-22).

2.1. Clinical specimens

Over the course of two governorates, Menofia and Qalyubia, a total of 140 samples were collected from freshly dead rabbits at various farms. Rabbits were of varying ages from 7 to 150 days and were all housed in cages. Rabbits showed the development of diarrhea with mortality rates ranging from 10 to 15 %, according to the age of the affected rabbit (Table 1). Between October 2020 and May 2021, macroscopic pathological abnormalities were recorded and samples, including liver and intestine, were obtained aseptically. These samples were preserved at -80 ˚C till usage to confirm the diagnosis by RT PCR.

Table 1 Epidemiological survey for the investigated rabbitry farms.

No.	Time of collection	Locality	Age/day	No. of examined animals /flock number	Mortality%
1	Oct. 2020	Sirs El-lyan	45	30 from 400	13%
2	Nov. 2020	Menofia	100	5 from 100	12%
3	Dec. 2020	Fesha El kopra	75	25 from200	15%
4	Jan. 2021	Shebin Elkom	150	10 from 500	12%
5	Feb. 2021	Qalyoub	120	40 from 150	10%
6	Mar. 2021	Tukh	7	10 from 100	11%
7	May 2021	Moshtohor	60	20 from 120	11%

2.2. Sample preparation

The samples were prepared by grounding them in PBS saline at a 1:3 concentration. Samples were frozen and thawed three times, centrifuged to remove the supernatant, and then filtered through a 0.22 m filter to remove debris and minimize background before being stored at -80°C until analysis by RT-PCR.

2.3. Reverse transcription-polymerase chain reaction (RT-PCR) for detection of suspected viruses

Using conventional RT-PCR, 140 samples were tested for the most prevalent rabbit diarrheal viruses. Rabbit hemorrhagic disease virus (RHDV) (Fahmy et al., 2010), rotavirus (Gómara et al., 2002), coronavirus (Lau Susanna et al., 2012) and Astrovirus (Todd et al., 2009).

Nucleic acid extraction was carried out in accordance with the manufacturer's instructions using the virus RNA/DNA nucleic acid extraction reagent included in the QIAamp viral RNA Mini kit (Qiagen, GmbH, Germany). Total RNA was frozen at -80°C for later use.

The reaction and program were adjusted according to the reference viruses and performed in a BIORAD® PCR system T100 thermocycler (BioRad, Hercules, California, USA). The RT-PCR products were identified by gel electrophoresis.

2.4. Sequencing and phylogenetic analysis for VP60 gene of RHDV strain:

Ultrapure 1.5% agarose was used for gel electrophoresis of 15 µl of RHDV primer (VP60) PCR products. (Invitrogen, Thermo Fisher Scientific, Germany) in 1×Tris-borate-EDTA (TBE) buffer at room temperature. The size of the product was measured using a 100 bp DNA ladder from Qiagen (Germany). A gel documentation system was used for imaging to detect PCR-amplified bands (Alpha Innotech, Biometra). Finally, using Automatic Image Capture Software, the data was examined (Protein Simple, formerly Cell Biosciences, San Jose, CA, USA). A QIAquick PCR Product extraction kit was used to purify PCR products for gene sequencing and phylogenetic analysis (Qiagen, GmbH, Germany). A Big Dye Terminator V3.1 cycle sequencing kit was used to accomplish the sequencing procedures (Perkin-Elmer), and purification was carried out by Centri-Sep spin columns (Thermo Fisher, Germany). The *VP60* sequences were obtained using a 3500xl genetic analyzer (Applied Bio-systems, Life technologies, Thermo Fisher, Germany). The Basic Local Alignment Search Tool (BLAST®) was used to search for similarities between sequences in the GenBank database and those in the research (Altschul *et al.,* 1990). The MegAlign module of Lasergene DNA-Star version 12.1 was used to calculate phylogenetic distances between the strains studied (Thompson *et al.,* 1994) MEGA7 was used to generate a phylogenetic tree with maximum composite likelihood, 1000 bootstrap replications, neighbor-joining, and maximum parsimony (Kumar *et al.,* 2016) and assembled sequences were deposited in the GenBank database.

3. RESULTS

3.1. PM findings:

The suspected diarrheic disease in rabbit farms was characterized by the presence of watery diarrhea and during PM examination of the carcass the most detectable lesion was hemorrhage nearly in most organs accompanied by poor blood coagulation led to the presence of clotted blood in blood vessels. Also, development of fluid with variable colors in small, large intestine and abdominal cavity. Presence of localized area of necrosis in different organs and splenomegaly. These lesions appeared in ages varied from 45 days to 150 days, while there were no PM lesions recorded in young rabbits of 7 days age (Table 2).

Table 2 Postmortem examination of dead rabbit

No.	Locality	Postmortem examination
1	Sirs El-lyan	Yellowish fluid within small and large intestine
2	Menofia	Sudden death, distended urinary bladder, greenish fluid in small intestine
3	Fesha El kopra	Yellowish fluid in abdominal cavity and small intestine
4	Shebin Elkom	Intestine was filled with fluid and gases
5	Qalyoub	The liver appeared yellowish-brown in color. Lungs were edematous and congested. Splenomegaly and presence of clotted blood in blood vessels and bloody intestine
6	Tukh	No PM
7	Moshtohor	Yellowish fluid in the pleural cavity, focal areas of necrosis and inflammation were found throughout parenchyma of various organs

3.2. Detection of viral causes using RT-PCR:

Molecular detection for the most probable viral causes of diarrhea in small rabbits (Corona virus- Rota virus-Astrovirus and Rabbit hemorrhagic disease virus). The results recorded in table (3) determined that RHDV was the only viral cause could be detected in the affected rabbits where it detected mainly in samples from rabbit farm located in Qalyoub, followed by farm at Sirs Ellyan, Menof, Shebin Elkom, Fesha Elkopra and Tukh.

3.3. Sequencing and phylogeny of RHDV strains based on VP60 gene:

Sequencing of RHDV in samples from rabbit herd of age 75 days located in Qalyoub using VP60 amplicon revealed a range of 97.1 to 87.6% nucleotide sequence identity (figure

3). The sequencing and phylogenetic analysis indicated that the amplicons were more closely related to RHDVb strains (Fig. 1). Sequences from the C-E region of VP60 were uploaded to GenBank for this strain and given the accession number OP265427.The nucleotide sequences of RHDV-2 strain and 20 RHDV sequences received from GenBank were aligned, and their details are provided in fig. (2) showing substitutions in nitrogenous bases in nucleotide sequence in several positions of VP60 of RHDV. Genetic analysis of RHV-Egypt-BenhaF954-2022 showed that it is clustered with G1.2/RHDV2/b strains with homology 97.1%. Homology with Egyptian classical strains were 96.6% with G1.1C/RHDV and 87.6% with G1.1a/RHDVa (Figs. 3-6).

Table 3 PCR survey against diarrhetic viruses

No.	Locality	Total No.	RHD virus	Rota virus	Corona virus	Astro virus
1	Sirs El-lyan	20	Positive (3)	Negative	Negative	Negative
2	Menofia	5	Positive (2)	Negative	Negative	Negative
3	Fesha El kopra	20	Positive (1)	Negative	Negative	Negative
4	Shebin Elkom	10	Positive (2)	Negative	Negative	Negative
5	Qalyoub	25	Positive (4)	Negative	Negative	Negative
6	Tukh	10	Positive (1)	Negative	Negative	Negative
7	Moshtohor	20	Negative	Negative	Negative	Negative
Total		110	13 positive samples	-	-	-

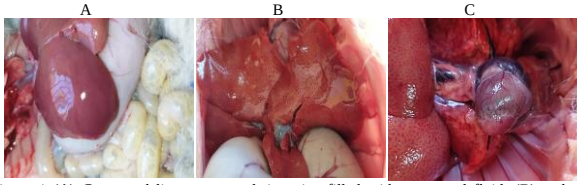

Figure 1 (A) Congested liver congested, intestine filled with gases and fluid. (B) and (C)focal necrosis of liver

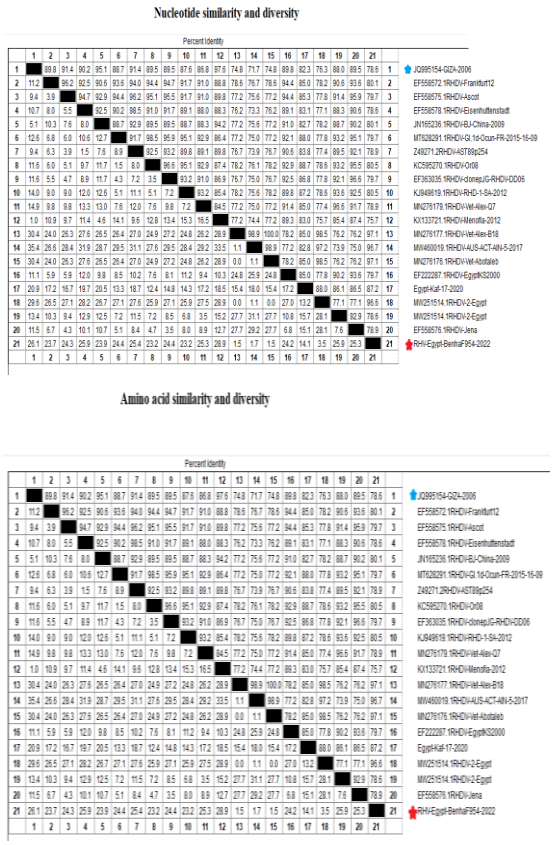

Figure 2 the identity % between identified strain with red star and the reference ones on GenBank

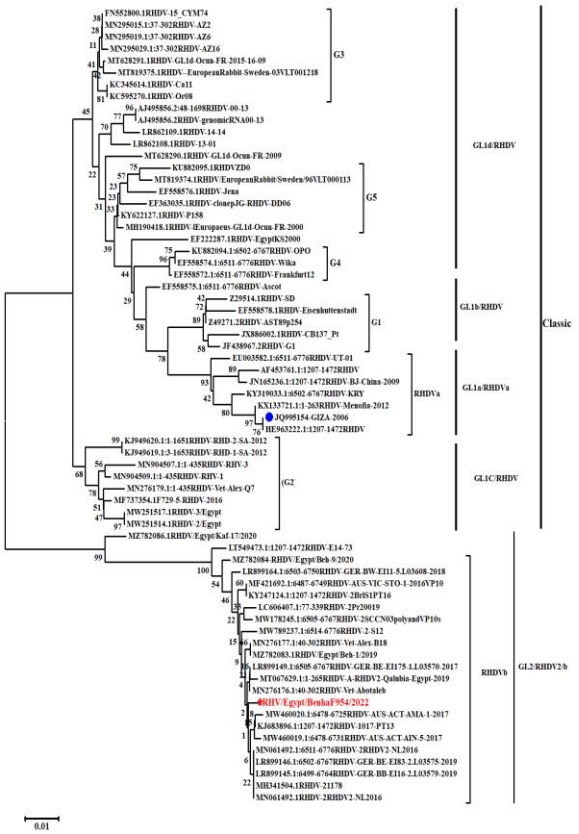

Fig. 3 Rabbit hemorrhagic disease virus phylogenetic tree based on partial nucleotide sequences (VP60 gene) and other randomly chosen strains from GenBank (MEGA 6-Neighbor-joining). Samples of our research are indicated in red font.

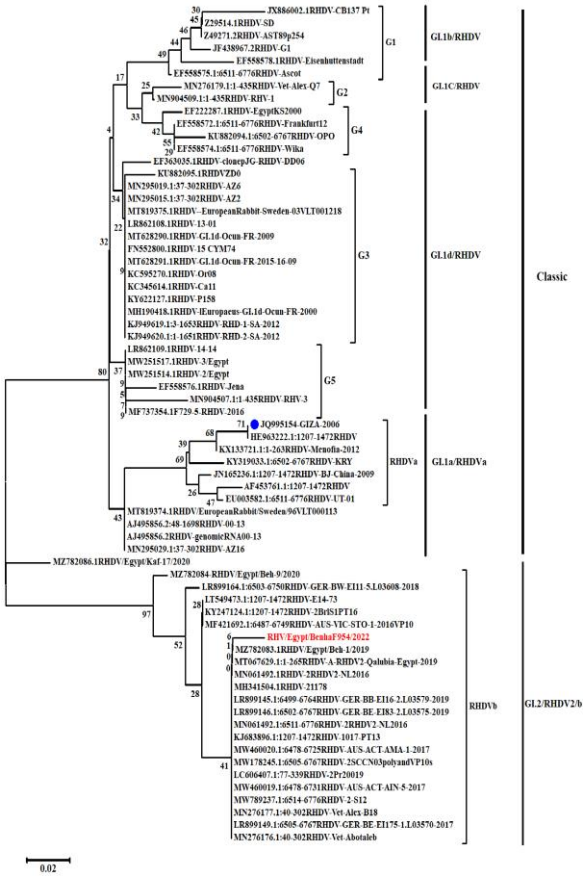

Fig. 4 Phylogenetic tree of rabbit hemorrhagic disease virus based upon partial Amino acids and other randomly selected strains from GenBank (MEGA 6-Neighbor-joining). Sample of our study are marked by red font.

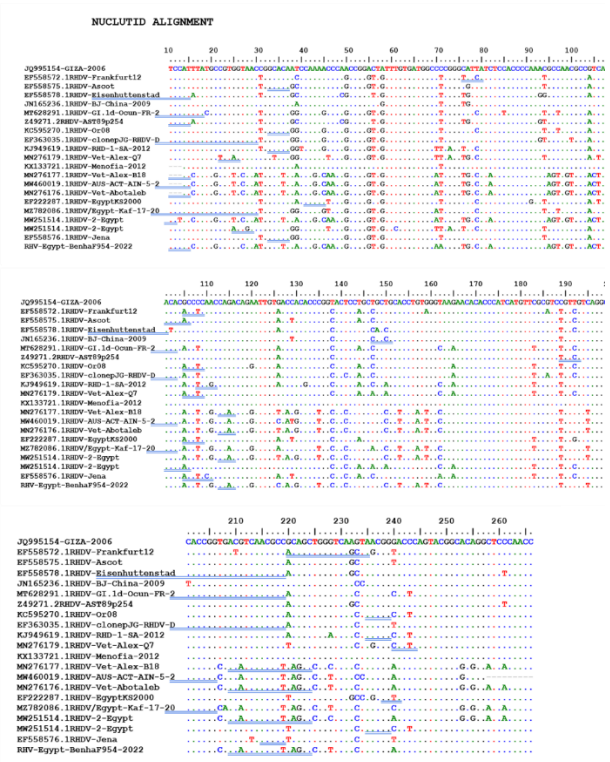

Fig. 5 Deduced nucleotide alignment of VP60 protein of the sequenced RHDV strain in comparison with other RHDV isolates selected from GenBank.

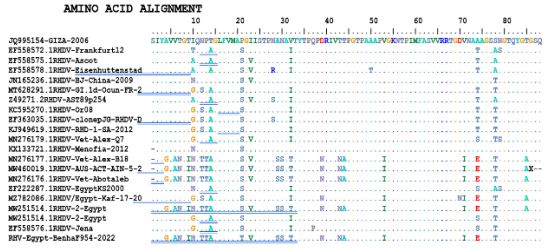

Fig. 6 Deduced amino acids of VP60 protein of the sequenced RHDV strain in comparison with other RHDV isolates selected from GenBank.

4. DISCUSSION

Most cases of rabbit enteritis probably had multiple etiological causes and viruses represent one of them (Cerioli and Lavazza, 2006). According to the results of the current study, the RHDV was the only detectable cause of enteritis in the examined young rabbits as recorded by Teifke et al. (2002), who reported that young rabbits, at the age of 4–8 weeks, or shortly after weaning, are highly susceptible to infection by RHDV. In this study, the clinical manifestation of diarrhea and sudden death reported in young and old rabbits (7 days until 150 days age).

Although RHDV might infect rabbits of any age, it rarely caused fatal illness in rabbits younger than 4 weeks old. This was because, between the ages of 4 and 12 weeks, the infant no longer benefited from age-based protection (Robinson et al., 2002). This described the reason for the absence of the PM lesions in rabbits with 7 days age in this study. As the subclinical form of RHDV occurs in rabbits younger than 6-8 weeks (OIE, 2019). Our analysis found that the bulk of instances occurred between October and March, which is in line with data from 1993 that showed a 26.7% to 100% death rate in rabbits between 14 and 16 weeks old in Upper Egypt (Minya, Assiut, and Sohag Provinces).

Other reports as El-Zanaty (1994) noted that RHDV was identified in rabbits during the spring of 1991 in Sharkia Province associated with 90% losses. Also, Erfan and Shalaby (2020) reported RHDV-2 variants in Lower Egypt provinces during the summer season, particularly in July.

Rabbit Hemorrhagic Disease Virus (RHDV) is characterized by an enlargement and discoloration of liver, swelling of spleen and kidneys hemorrhage in the upper respiratory tract and lungs, (Abrantes et al., 2012). This comes as the same with the P\M examination in the current findings, showing presence of yellowish fluids in the abdominal cavities and in the intestine with discoloration of the liver. This observation like that recorded by El-Nahas (2011) where the pathological findings demonstrated discolored liver and presence of bloody mucous in the intestine. and with Carvalho et al. (2016), who observed Hemorrhagic tracheitis, hepatic congestion, friable liver with necrosis and congestion of lung in rabbit infected with RHDV2. In this study, 13 cases were detected as positive for RHDV by RT-PCR and partial comparing sequencing utilizing a 538-bp fragment of the VP60 gene.

These findings agreed with those detected by Le Gall-Reculé et al. (2017), who claimed that VP60 was the most effective and general screening tool for rabbit and hare caliciviruses and the best target for RT-PCR assays. Hall *et al.* (2018) stated that all rabbit Lago viruses in Australia were analyzed using the viral VP60 gene.

Viral strains are categorized and typed based on their primary capsid protein, Vp60. To date, three major viral subtypes have emerged among the many RHDV strains: RHDV ("classical RHDV"), the antigenic variant 5RHDVa, and the newly emerged virus RHDV2 (also called RHDVb). RHDV2 is a different serotype than the classic RHDV and RHDVa (CFSPH, 2016). Based on our sequencing analysis, we know that our strain is related to RHDV2 strains, which were not previously isolated in Egypt until 2020, when they were discovered in the governorates of lower Egypt by Erfan and Shalaby (2020). Some authors claim that RHDV2 is now the most common strain of the virus in Europe, replacing older RHDV strains and being responsible for the vast majority of reported cases in both wild and domestic rabbits (Mahar et al., 2018).

The phylogeny of the isolated strain shows that it related to RHDV2 with nucleotide identities ranging from 97.1 to 96.6% compared to the available RHDV2 strains in GenBank. Genetic analysis of RHV-Egypt-BenhaF954-2022 showed that it is clustered with G1.2/RHDV2/b strains with 97.1% homology to MN276176 and MW 276177 and 96.6% homology G1.1C/RHDV as MW 251514 RHDV-2/Egypt classical Egyptian RHDV strains and with lowest homology with JQ99515-Giza-2000 with 87.6% belong to G1.1a/RHDVa cluster. This finding corroborated Erfan and Shalaby's (2020) and Abido et al. (2020) claiming that RHDV-2 was detected in Egypt's Delta governorates.

5. CONCLUSIONS

Finally, the genetic diversity between the obtained strain and other reference RHDV strains indicates the possibility of antigenic differences and the need for additional research on the virus's molecular and epidemiological characteristics.

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