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# Genetic and immunogenic studies of variant Rabbit Hemorrhagic Disease Virus under controlled laboratory conditions

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**Abstract**

Rabbit viral hemorrhagic disease (RHD) is a highly contagious viral disease that causes severe economic losses in rabbits. Safe and potent oil adjuvant vaccine against RHDV is required to control the disease. In the present study, RHDV was confirmed by RT-PCR in 5/20 rabbit farms suffered from fever, depression, nervous manifestation, hemorrhage, sudden death, splenomegaly, and pneumonia. The VP60 protein partial nucleotide sequence analysis for the 5 RHDVs showed 96.6% to 97.3% identity with the variant RHDVa/G6 viruses. To develop a potent RHDV vaccine, safety, dose-response and potency of three inactivated vaccine formulations were studied. Safety of developed vaccine formulations was assessed using 20 rabbits divided into two groups (S-1 and S-2) that received 1× and 2× doses via intramuscular routes, respectively. The potency experiment was conducted using 40 rabbits divided into 4 groups (n= 10/ group). Three vaccinated groups I, II, and III that received inactivated RHDV vaccine containing either 800, 1000, or 1200 HAU/dose, whereas, group (IV) was a placebo control. Seroconversion was evaluated on a weekly basis post-vaccination (PV) using haemagglutination inhibition (HI) test. On day 28 PV, all groups were challenged with 100 $LD_{50}$/rabbit using ME/RHD-01/2017 virus via the intranasal route. The tested RHDV vaccines were safe in both single and double overdose. The three tested formulas induced detectable humoral antibody titers at W1 PV and reached $\geq 9$ $Log_2$ by W4 PV. Generally, group III received 1200 HAU/dose showed a significantly higher antibody response ($P \leq 0.05$) in all weeks PV. Protection rates in vaccinated rabbits were 80%, 100%, and 100% in groups received 800, 1000 and 1200 HAU/dose, respectively. In conclusion; the developed inactivated RHDV vaccine showed superior protective, a rapid humoral immune response against virulent RHDVa/G6 from W1 PV. Additionally, the vaccine dose of 1200 HAU/dose of RHDV antigen was found to be protective against variant RHDVa/G6 virus.

***Keywords:*** *Rabbit Hemorrhagic Disease Virus, Genetic analysis Immunogenicity, vaccine, Challenge and Potency*

## Introduction

RHD is highly contagious acute worldwide viral disease of rabbits **(Abrantes *et al.*, 2012)**. The causative agent is RHDV which belongs to the genus *Lagovirus* within the family *Caliciviridae*. The virus is naked icosahedral, positive sense RNA virus **(Ismail *et al.*, 2017)**. RHDV was first reported at 1984 in China **(Liu *et al.*, 1984).** Subsequently, the disease rapidly spread to other countries **(Gregg *et al., 1991).*** In Egypt, RHDV was first reported in 1991 **(Ghanem and Ismail, 1992)**. In spite of vacci-

nation programs, RHD is still representing a threat in rabbit production farms due to high morbidity and mortality rates **(Alboghdady and Alashry, 2010)**. The clinical picture of RHD is characterized by fever, depression, nervous manifestation, haemorrhage and sudden death **(Trzeciak-Ryczek *et al.*, 2015).** Post mortem pathological changes include hepatitis, splenomegaly and pneumonia **(Embury-Hyatt *et al.*, 2012).** The disease causes high mortality rates of 70–100% in adult rabbits while young kits are sub-clinically infected **(Dalton *et al.*, 2012).** In 1992, trials of RHDV vaccine preparation were adopted by **(Salem, 1992)** who prepared inactivated formalized tissue vaccine from liver and lung suspensions of rabbits which were previously infected with RVHD virus, rabbits were protected by seven days after vaccination and immunity lasted for more than two months, also **(Daoud *et al.*, 1998)** succeeded to produce an inactivated RHDV vaccine from the local isolate. The present study aimed to conduct genetic analysis of recent RHDV, evaluate the safety, dose response and potency of newly-developed inactivated oil adjuvant (ISA70) vaccine against RHDV.

## Materials and Methods

### Field samples:

A total of 5 rabbits were sampled from 20 different rabbit farms from 3 governorates "Menofia, Shrakia and Kafer El-Sheikh" during period from 2016 to 2017. All tested rabbit farms were vaccinated using commercially available formalin based RHD vaccine, aged ≥ 6 months and suffered from high mortality. Liver samples were collected separately and homogenated using sterile PBS in a ratio of 1gm/5ml (W/V) under complete sterile condition (***OIE, 2012)***. The liver homogenates were centrifuged at 4000 rpm for 30 min and the supernatants were collected and preserved in -80°C for further testing further testing.

### Molecular characterization of RHDV

**One-step RT-PCR:** Viral RNAs were extracted from liver suspensions with RNeasy (QIAGEN, Germany). Five microliters of purified RNA extracts were used in a 25 µl reaction mixtures using the Thermo Scientific Verso One-Step RT-PCR Kit (ABgene ® UK). The RHDV upstream (P33: 5‟- CCACCAC-CAACACTTCAGGT -3‟) and downstream (P34: 5‟CAGGTTGAACACGAGTGTGC-3") specific primers were used according to ***(Vende et al., 1995).*** 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 (538 bp) were visualized by electrophoresis on a 1% agarose gel in 1X Tris-acetate–EDTA (TAE) buffer (40 mM of Tris and 2 mM of EDTA, pH 8.0) and photographed under 304 nm UV light (UV Transilluminator, Major Science).

**Nucleotide sequence analysis:** PCR products sequencing was performed in both directions with virus-specific primers. Sequences were analyzed using the 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 obtained **453** nucleotide sequences of the VP60 protein. The detected sequences were compared with others deposited in GenBank by multiple alignments with the Clustal W included in Bioedit software. Phylogenetic relationships were evaluated by the Neighbor-Joining method using the MEGA version 6 software with 1000 bootstrap replications **(Tamura *et al.*, 2013)**.

### Vaccine preparation

### RHD Virus

ME/RHD-Menofia/2012 strain (KX133721.1) was selected for RHDV for vaccine master and working seed and antigen production. The isolated virus based pathogenicity and purity were confirmed according to the OIE manual **(OIE 2012)** (data not shown). The virus stock was prepared from 2-3 month non-vaccinated rabbit liver and spleen homogenates inoculated with a dose of $10^2$ $LD_{50}$/rabbit via the intramuscular route. Harvested livers and spleen of infected rabbits died between 24 and 72 hours post-inoculation were minced in 1/10 (w/v) sterile PBS, pH 7.2–7.4, and the mixture was homogenized using ultra-high homogenizer at speed of

20000 rpm for 20 min in a cold environment. The mixture is then treated with 2% chloroform (18 hours at 4°C), followed by centrifugation at 6000 g for 1 hour at 4°C. The virus was titrated and the titer of the liver suspension was $10^{6.5}LD_{50}$/ml and 16384 HAU/25ul.

**Vaccine preparation**

The RHD vaccines formulations were prepared at Middle East for Veterinary Vaccines (ME VAC) laboratories. Water in Oil (W/O) inactivated vaccine emulsion was prepared from ME/RHD-Menofia/2012 strain. The virus was inactivated using 0.3% formalin. The completion of inactivation was assured by inoculation of the inactivated virus suspension in susceptible non-vaccinated 2-3 old rabbits for 3 blind passages. The inactivated virus suspension was then blinded with the oil Adjuvant Montanide ISA70 (Seppic, France). Three different RHD vaccine formulations were prepared using 3 different dose concentrations (800, 1000 and 1200 HAU/rabbit dose).

**RHDV Vaccine evaluation and testing**

**Experiment-I: Vaccine Safety study**

The safety of the vaccine formulation containing the highest antigen dose (1200 HAU/dose) was evaluated. A total of 30 non-vaccinated 3-month-old rabbits were used for safety testing. Animals were allotted into 3 different equal groups (Table 1). Group S-I were vaccinated I/M with 1× dose, group S-II was vaccinated I/M with 2× dose, and group S-III received placebo emulsion containing PBS. The safety was assessed based on clinical sings and fever. At 14 and 21 days PV, 3 rabbits form each group were sacrificed humanly for checking the vaccine adjuvant release and absorbance from site of inoculation, local reactions at site of injection, and postmortem examination of liver, lung and other internal organs for any deviation compared to the placebo control group.

**Experiment-II: Vaccine dose response and efficacy study design**

A total of 40 non-vaccinated 3-month-old rabbits were used for the dose response and effectiveness study. The 40 rabbits were allotted in 4 equal groups (Table 2). The 3 vaccination groups received 800, 1000, and 1200 HAU/dose of RHDV vaccine in group I, group II, and group III, respectively. Rabbits in group IV received a placebo W/O emulsion and served as non-vaccinated controls.

The 4 rabbits groups (I, II, III and IV) were then challenged at $4^{th}$ week PV using $100LD_{50}$/rabbit of the virulent RHDV (ME/RHD-01/2017) via intramuscular (I/M) route **(OIE, 2012)**. The effectiveness was evaluated based on morbidity, mortality, and protection rates.

**Serological monitoring using hemagglutination inhibition test**

Serum samples of individual rabbits collected weekly for 4 weeks PV were inactivated for 15 minutes at 56°C ***(Capucci et al., 1996).***. The sera were diluted as two-fold serial dilution and incubated with equal volume of 8 HAU RHDV antigen in a sealed round-bottom microtiter plates at room temperature. Washed human type O RBCs (0.75 concentration) were then added and plates incubated at room temperature. The antibody positivity titer was $\geq 3\log_2$. Seroconversion rate was calculated using following formula = (No of Positive individual/total tested sample) X 100.

**Table (1).** Experiment No. 1 RHDV vaccine Safety test design

| Group | No. of animals | Vaccine | Dose | Route |
|---|---|---|---|---|
| **Group S-I** | 10 | RHDV 1200HAU/ dose | 1× (1ml) | Intramuscular |
| **Group S-II** | 10 | | 2× (2ml) | |
| **Group S-III** | 10 | MEVAC Placebo | 1 ml | |

[1]MEVAC RHD trial No.3 (1200 HAU/dose) were used for safety test
All rabbits were monitored on daily basis PV for clinical sings and fever.

then added and plates incubated at room temperature **(OIE, 2102)**. The antibody positivity titer was $\geq 3\log_2$. Seroconversion rate was calculated using following formula = (No of Positive individual/total tested sample) X 100.

**Table (2).** RHDV vaccine dose response and efficacy study design

| Group (N=10) | Vaccine[1] | | Route | Challenge[2] | |
|---|---|---|---|---|---|
| | Formula | HAU/dose | | Virus | Dose/Route |
| **Group I** | RHD1 | 800 | Intramuscular | ME/RHD-01/2017 | 100 $LD_{50}$/ rabbit /Intramuscular |
| **Group II** | RHD2 | 1000 | | | |
| **Group III** | RHD2 | 1200 | | | |
| **Group IV** | Placebo | PBS | | | |

[1]Vaccination was conducted using full vaccine dose 1ml via intramuscular route
[2]challenge test were conducted 4 weeks post vaccination. All rabbits were monitored on daily basis PV and post-challenge (PC), weekly individual blood samples for 4 weeks.

## Results

### RHD virus detection

The RHDV was detected only form 5 tested farms from both Menofia and Sharkia governorates. All the positive rabbit farms were vaccinated and suffered from typical RHD clinical signs. Mortalities ranged between 50 and 70%. The RHDV was isolated from the positive PCR samples and were molecularly characterized. The viruses were assigned as ME/RHD-01/2016, ME/RHD-02/2016, ME/RHD-01/2017, ME/RHD-02/2017 and ME/RHD-01/2018.

### RHDV VP60 protein sequence analysis

The partial sequences of VP60 protein of different RHD viruses showed 100% identity with the previously reported RHDV local isolate strain (Menofia/2012) (KX133721.1) and were closely related to the SHAH 2015 virus (KY316900) (96.6-97.3%). Phylogenic and nucleotide identity analyses indicate that these viruses are related to the variant RHDa/G6 viruses isolated in china 2006, 2009, 2011 and also from Iowa, USA 2000 (94.5-95.6%). However, their identity with other genotypes 1, 2, 3, 4 and 5 were (92.3-93%), (93-94%), (90.9 -91.1%), (90.2-90.5%), and (90.9-91.4%), respectively **(Figure 1 and Table 4).**

### RHDV vaccine safety

The safety study showed that the new prepared oil adjuvanted RHDV vaccine was safe when administered as single full dose or double dose. There was no local or generalized reaction after inoculation of rabbits with single and double doses **(Table 3).**

**Table (3).** RHDV Vaccine Safety in 3-month-old rabbits

| Group[1] | Vaccine release | Clinical sings | Local reaction | Ear Temp °C | Postmortem examination |
|---|---|---|---|---|---|
| Group S-I | 100% | Healthy | None | 38.9+0.3 | No change |
| Group S-II | 100% | Healthy | None | 39.3+0.4 | No change |
| Group S-III | 100% | Healthy | None | 39.3+0.3 | No change |

[1]Group S-I vaccinated with full dose (1ml) contains 1200 HAU/dose, group S-II vaccinated with 2× full dose (2ml) contains 2400 HAU/dose), and group S-III received placebo emulsion vaccine (1ml) that contains. All rabbits were monitored on daily basis PV for clinical sings and fever.

**Vaccine dose response and potency results**

**Humoral antibody response Post-vaccin-ation**

In all vaccinated rabbit's groups, detectable humoral antibody responses were observed by the first week PV **(Table 5).** The mean HI log2 titers (6.2 + 0.4) and seroconversion rate (100%) were significantly higher in rabbits that had received the 1200 HAU doses compared with those that had received the 800 and 1000 HAU doses. Starting from W2 PV, the HI antibody titers in GIII that had received 1200 HAU was significantly higher (9.2+0.7) than those of groups that had received 800 and 1000 HAU (7.2 +0.4 and 8 +0.8 respectively). Moreover G III that had received 1200 HAU showed significantly ($P \leq 0.05$) higher antibody titers at W3 and W4 PV compared with other groups. The PV seroconversion rate (titer > 4 log 2) was reach 100% in GI and GII at W3 Post-vaccination while GIII showed 100% seroconversion starting from the second week PV **(Table 5, Figure 3).**

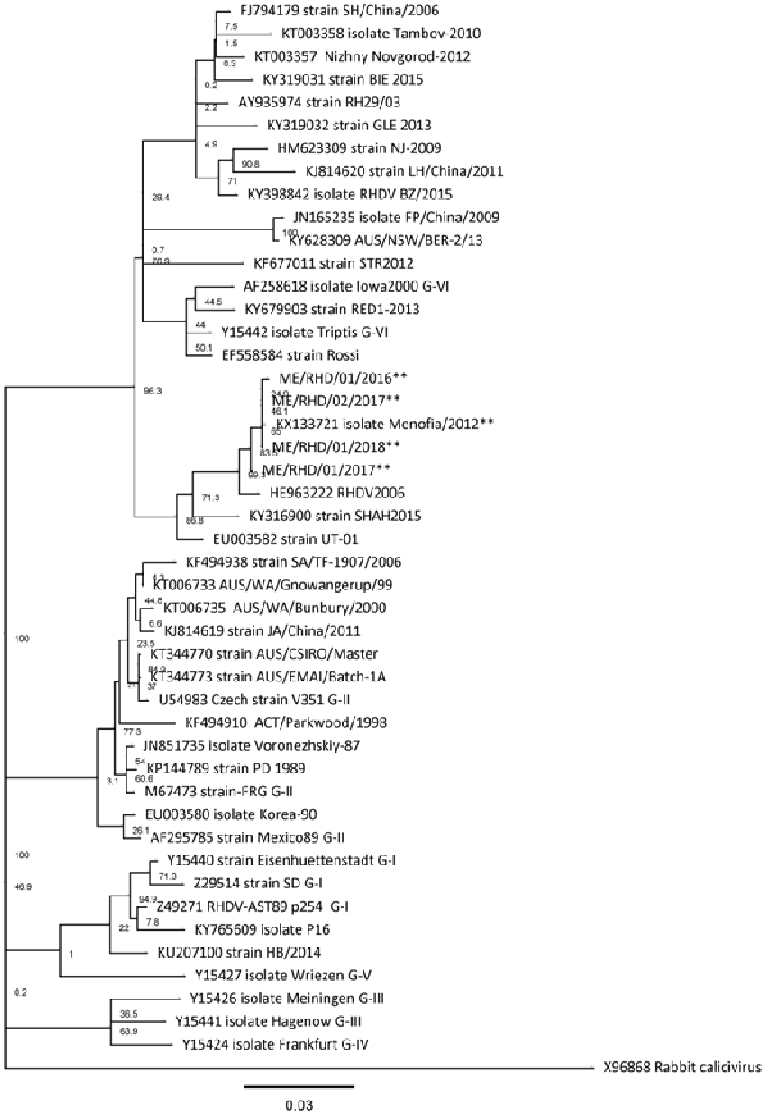


**Figure (1).** Phylogenetic tree based on VP60 protein partial nucleotide sequence of RHDV local isolate strain (Menofia/2012 KX133721.1) and previously published sequences. Marked strain (**) refers to local isolate under study. Representative strains from different genotypes were included. Phylogenetic relationships through a bootstrap trial of 1000 were determined with the MEGA version 6 using the Clustal W alignment algorithm and neighbor-joining method for tree construction.

**Table (4).** Nucleotide sequences alignment of VP60 gene of RHD virus strains in comparison to other different strains.

| Strains | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| KX133721 Menofia/2012** | | 100 | 100 | 100 | 100 | 98.8 | 97 | 97.1 | 95 | 92.3 | 93.1 | 90.5 | 91.1 | 91.1 | 81.3 |
| ME/RHD/01/2016** | | | 100 | 100 | 100 | 98.8 | 96.9 | 97.1 | 94.9 | 92.4 | 93 | 90.3 | 90.9 | 90.9 | 81.1 |
| ME/RHD/01/2017** | | | | 100 | 100 | 99.3 | 97.5 | 97.1 | 95.6 | 93.1 | 94 | 90.8 | 91.5 | 91.5 | 81.3 |
| ME/RHD/02/2017** | | | | | 100 | 98.9 | 96.7 | 97.1 | 94.5 | 93 | 93.4 | 90.5 | 91.4 | 91.4 | 81.3 |
| ME/RHD/01/2018** | | | | | | 99.3 | 97.3 | 97 | 95.3 | 92.9 | 93.6 | 90.2 | 90.9 | 91.1 | 81.2 |
| HE963222 RHDV2006 | | | | | | | 97 | 96.6 | 95 | 92.5 | 93.3 | 90.7 | 91.3 | 91.3 | 81.3 |
| EU003582 strain UT-01 | | | | | | | | 97.5 | 96.4 | 93.8 | 94.2 | 92.3 | 93.1 | 92.9 | 83.1 |
| KY316900 strain SHAH2015 | | | | | | | | | 94.1 | 93.6 | 93.6 | 91.2 | 92.1 | 92.4 | 83 |
| AF258618 isolate Iowa2000 G-6 | | | | | | | | | | 93.8 | 93.3 | 91.1 | 91.5 | 91.5 | 82.3 |
| Y15440 Eisenhuettenstadt G-1 | | | | | | | | | | | 97.2 | 94.8 | 95 | 95 | 83.1 |
| AF295785 strain Mexico89 G-2 | | | | | | | | | | | | 95.6 | 95.8 | 95 | 83.7 |
| Y15424 isolate Frankfurt G-4 | | | | | | | | | | | | | 96.4 | 94.8 | 83.5 |
| Y15441 isolate Hagenow G-3 | | | | | | | | | | | | | | 95.4 | 83.3 |
| Y15427 isolate Wriezen G-5 | | | | | | | | | | | | | | | 82.9 |
| X96868 Rabbit Calicivirus | | | | | | | | | | | | | | | |

**Table (5).** Post-vaccination humoral antibody response titers (Mean±SD) in different vaccinated rabbits groups

| Weeks PV[2] | Mean HI $log_2$ titer (+SD)[1] | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Group I | | Group II | | Group III | | GIV | |
| | Titers | Pos.(%) | Titers | Post.(%) | Titers | Post.(%) | Titers | Post.(%) |
| W0 | 3 (+0.7) [a] | 10% | 3.1(+0.5)[a] | 10% | 3.2 (+0.4) [a] | 10% | 3.2 (+0.5)[a] | 10% |
| W1 | 5 (+0.7) [a] | 50% | 5 (+1.2) [a] | 80% | 6.2 (+0.4) [b] | 100% | 2.6 (+0.5) [c] | 0% |
| W2 | 7.2 (+0.4) [a] | 100% | 8 (+0.8) [b] | 100% | 9.2 (+0.7) [c] | 100% | 2.8 (+0.5) [d] | 0% |
| W3 | 9.4(+0.5)[a] | 100% | 9.6(+0.5)[a] | 100% | 9.6 (+0.9) [a] | 100% | 2.8 (+0.5)[b] | 0% |
| W4 | 9.8 (+1.4)[a] | 100% | 11 (+0.5)[b] | 100% | 11.5 (+0.7)[c] | 100% | 2.8 (+0.5) [d] | 0% |

[1] Mean HI $log_2$ titer at time of testing and mean Standard Deviation (SD) between individual tested rabbits titers in each group. [2]Weeks PV; Weeks Post-vaccination. Group I (vaccinated with full dose 1ml that contains 800 HAU/dose). Group II (Vaccinated with full dose 1ml that contains 1000 HAU/dose). Group III (Vaccinated with full dose 1ml that contains 1200 HAU/dose). Group IV (Vaccinated with placebo vaccine).

[a, b, c, d] Different superscript litter in each raw donate the presence of statistical significance ($P \leq 0.05$)

GI (vaccinated with full dose 1ml that contains 800 HAU/dose). GII (Vaccinated with full dose 1ml that contains 1000 HAU/dose). GIII (Vaccinated with full dose 1ml that contains 1200 HAU/dose). GIV (Vaccinated with placebo vaccine).

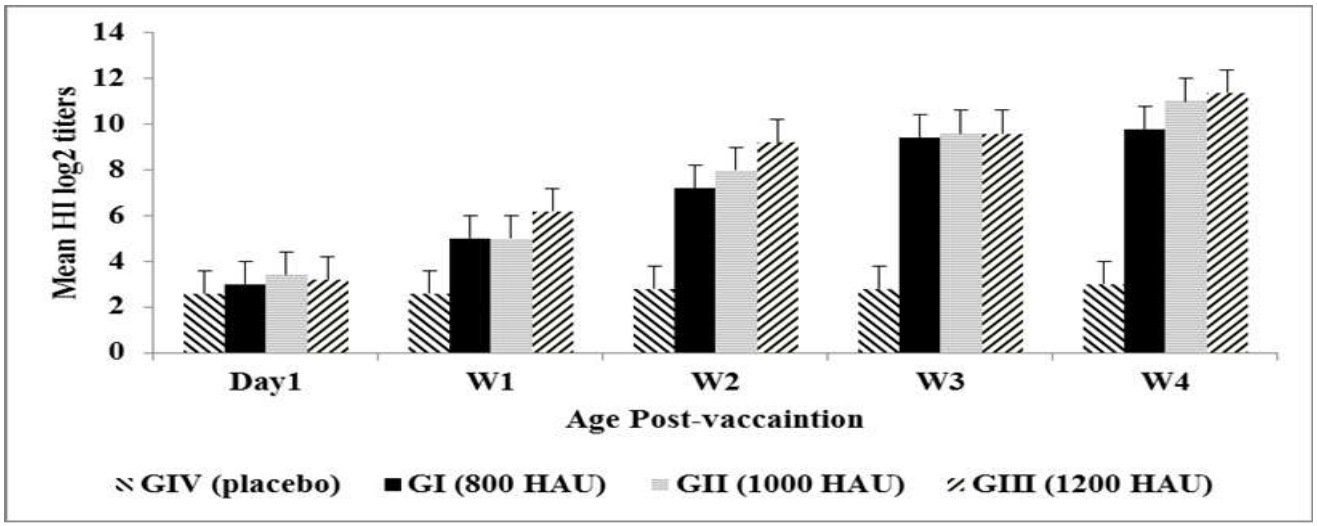


**Figure (3).** Results of weekly post vaccination indifferent vaccinated rabbits groups

**Vaccine challenge results**

Generally, rabbits that received the highest antigen concentration (i.e. 1200HAU/dose) showed superior protection level (100%) in addition to the best clinical protection (Morbidity 0% and Mortality 0%). While the other two vaccinated groups (GI and GII) have showed different clinical manifestation from 2nd day PC, where, morbidity reached 50% & 20%, and mortalities were 20% & 0%, respectively. The placebo vaccinated control group showed 100% morbidity 100% and 83.3% of rabbits died (Table 6).

**Table (6).** Daily observation and morbidity, mortality and protection rates post challenge

| Group (N=10) | Day Post-challenge[1] | | | | | | | | | | Morbidity% | Mortality % | Protection% |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | | | |
| GI | 0 | 1D[2] | 1D | 2 | 1 | 0 | 0 | 0 | 0 | 0 | 50 | 20 | 80% |
| GII | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 20 | 0 | 100 |
| GIII | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 100 |
| GIV | 2D | 5D | 2D | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 100 | 83.3 | 16.7 |

[1]All groups were challenged at W4 post-vaccination using virulent RHDV (ME/RHD-01/2017) via intramuscular (I/M) route inoculation using 100LD50/0.5ml per rabbit. [2] D; dead bird

Morbidity %= (number of diseased rabbits/total) X 100.

Mortality %= (number of dead rabbits/total) X 100.

Protection %= (number of survival rabbits/total) X 100.

Group I (vaccinated with full dose 1ml that contains 800 HAU/dose). Group II (Vaccinated with full dose 1ml that contains 1000 HAU/dose). Group III (Vaccinated with full dose 1ml that contains 1200 HAU/dose). Group IV (Vaccinated with placebo vaccine).

**Discussion**

RHD is a highly fatal disease affecting both domesticated and wild rabbits of the genus Oryctolagus cuniculus. RHD was firstly identified in China in 1984 **(Liu *et al.,* 1984),** Korea 1987 **(Park *et al.,* 1987)** later on, the disease appeared on Italy 1986 **(Cancellotti and Renzi, 1991)** and subsequently spread to Europe and worldwide **(Argüello Villares *et al.,* 1989); Villafuerte *et al.,* 1995; (Delibes Mateos *et al.,* 2008).** Moreover, RHDV outbreaks were reported in North Africa **(Morisse *et al.,* 1991),** Americas (Mexico), in 1988 **(Gregg *et al.,* 1991).**

In Egypt, though the RHD was reported since 1991 **(Ghanem and Ismail, 1992),** there is very limited literature about the epidemiology of the virus in Egypt due to lacking of organized national surveillance and epidemiological studies for rabbits diseases. The RHD classical clinical and postmortem pathological pictures were reported as fever, depression, nervous manifestation, haemorrhage, sudden death, hepatitis, splenomegaly and pneumonia **(Embury-Hyatt *et al.,* 2012; Trzeciak-Ryczek *et al.,* 2015; Bazid *et al.,* 2015).**

In the current study, the typical disease picture was reported in the 5 confirmed RHD positive farms during the period from 2016 to 2018 with mortality rates of 50-70%. Higher mortality rates of 70-100% were previously reported in adult rabbits with subclinical infection in young kits **(Dalton *et al.,* 2012).** Although RHDV is a RNA virus, the RNA and protein sequences of different isolates of RHDV are highly conserved **(Milton *et al.,* 1992; Nowotny *et al.,* 1997; Le Gall *et al.,* 1998); Asgari *et al.,* 1998).** However, many phylogenetic studies have evaluated the genetic variation between different RHDV isolates collected from different countries over a period of several years. Hence, the possibility of distribution

and genetic grouping of RHDV isolates based on year to year evolution was suggested based on studying over 95 RHDV isolates from 18 different countries representing the period from 1987 to 1995 **(Nowotny *et al.,* 1997; Le Gall-Reculé *et al.,* 2003).**

Recent genetic analyses based on the RHDV VP60 gene, antigenic and epidemiological data have indicated the existence of three main RHDV groups including "classical RHDV" containing 5 genogroups (G1–G5) reported in more than 40 countries **(Le Gall-Reculé *et al.,* 2003),** the subtype RHDVa/G6 have been reported in Europe on 1996 **(Capucci *et al.,* 1998; Schirrmeier *et al.,* 1999)** as new variant, and the "new" RHDV (provisionally called RHDV2 or RHDVb) emerged in France in 2010 in wild and farmed vaccinated rabbits and rapidly spread in Europe and the Mediterranean basin (Malta and Tunisia), and also in Australia in 2015 **(Dalton *et al.,* 2012; Le Gall -Reculé *et al.,* 2013).**

In the current study genetic analysis of VP60 protein of the 5 PCR positive RHD viruses are closely related to the local isolate strain (Menofia/2012) (KX133721.1) and are related to the SHAH 2015 virus (96.6-97.3%). These viruses belong to the subtype RHDVa/G6 G-VI viruses (94.5-95.6%) which were recently isolated from France, Europe, Oceania, Asia and Americas **(Capucci *et al.,* 1998)** and **(Schirrmeier *et al.,* 1999).** Meanwhile, the detected virus's identities to the previously reported genotypes I to V ranged between 90.2 to 94% (Figure 1 and Table 3). These results confirm the circulation of RHDVa/G6 new variant stains in Egypt and may explain the current continuous RHDV outbreaks and vaccination failures of available vaccines.

Previous studies adopted different trials of oil-based RHDV vaccine preparations using different local isolate showed that vaccinated rabbits can be protected as early as seven days after vaccination and an immunity period lasted for two months. **(Salem, 1992; Daoud *et al.,* 1998).** Notably, the potency of different commercial non-oil based RHD vaccines against RHDV showed high potency, protecting against challenge by 3 weeks PV. Conversely, the detected antibody titers of these vaccines were low at the 1st week PV indicating a fair degree of protection can be expected before 21 days post vaccination **(Šmíd *et al.,* 1991).** Considering the advantages of genetic relatedness and the better efficacy of oil-based vaccines, we aimed to develop a new homologous oil-based RHDV vaccine using the RHDVa/G6 related virus (ME/RHD-Menofia/2012 (KX133721.1). Safety, dose-response, and efficacy studies were conducted to evaluate the newly developed vaccine.

The safety and absorbance of the injected vaccine was confirmed in rabbits using double dose with complete release of the oil from the site of injection after 14 & 21 days PV. The results confirm the suitability of developed oil-based vaccine for field application in rabbit farms with no influence on the quality of the meat and fur of vaccinated rabbits ted rabbits **(Mitro and Krauss, 1993; Huang, 1991).** The new RHDV inactivated vaccine emulsion (W/O) was formulated using Montanide ISA 70 adjuvant (30/70) using 3 different doses of new RHD vaccine doses 800, 1000, and 1200 HAU/dose. The post-vaccination humoral antibody response against RHDV using HI test showed that the 3 new oil-based formulations induced detectable humoral antibody response as early as 1 week PV (≥5.0 Log2) with a significantly higher titers (6.2±0.4 log2) in groups received the maximum antigen concentration of 1200 HAU/dose ($P \leq 0.005$) and 100% seroconversion rate starting from week 1 PV compared to other groups. These data further confirm that RHDV oil-emulsion vaccine induce higher and longer HI antibody titres and duration of immunity in rabbits compared to non-adjuvant vaccine **(Šmíd *et al.,* 1991; Mitro and Krauss, 1993; Huang, 1991).**

Accordingly, the same group (1200 HAU/dose) showed superior clinical protection with 0% morbidity and 100% protection level against virulent RHDV experimental challenge. The other two groups received 800 and 1000 HAU/dose of the vaccine showed variable clinical manifestation by 2nd day PC.

Both serological monitoring and challenge results indicate that the RHDV vaccine formulation contain 1200 HAU/dose was able to induce rapid immune response and to protect 100% against both virulent RHDV induced morbidity and mortality. Meanwhile, a dose of 1000 HAU/dose showed 100% protection rate (Table 4).

**In conclusion,** the current study further confirms the circulation of the variant RHDVa/G6 genotype in Egypt indicating the necessity of adopting a national epidemiological surveillance for RHDV to monitor the new genetic and antigenic variant viruses' outbreaks. The newly developed W/O inactivated RHDV vaccine using genetically related RHDV strains with a minimum dose of ≥1000 HAU/dose can induce rapid and protective immune response against the virulent RHDVa/G6 in addition to its safe application in rabbits. Further studies are required to evaluate the protection of the developed vaccine and other commercially available vaccines against the heterologous challenge especially the new RHDVa/G6 genotype.

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