



# Immunogenicity and Efficacy of a Bivalent Inactivated Vaccine against Rabbit Hemorrhagic Disease Virus

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## ABSTRACT

Rabbit hemorrhagic disease is a fatal threat to rabbits that causes sustainability problems and substantial economic losses. The aim of the current study was to compare the immuno-enhancing effects of a bivalent inactivated rabbit hemorrhagic disease virus (RHDV) vaccine adjuvanted with Montanide with an inactivated RHDV vaccine with an aluminum hydroxide gel. Montanide incomplete seppic adjuvant 71 VG was prepared as an oil emulsion, and several batches adjuvanted with an aluminum hydroxide gel were prepared. Then, 250 New Zealand rabbits aged 6 weeks were randomly allocated to three groups. Group 1 was subjected to the bivalent inactivated RHDV adjuvanted with an aluminum hydroxide gel, Group 2 received the oil-emulsion vaccine adjuvanted with Montanide, and Group 3 was left unvaccinated as a negative control group. Efficacy was determined using a hemagglutination inhibition test, and resistance was determined using virulent RHDVa and RHDV2. The clinical signs included sudden death, nervous manifestations, aimless running, lateral recumbence, and crying before death. The mortality rates were recorded at 3 weeks, 3 months, 6 months, and 12 months after vaccination. In addition, blood samples were collected on the first day as well as 1, 2, 3, 4, 6 weeks post-vaccination (WPV), and 2, 3, 4 month post-vaccination (MPV) until 12 MPV. Serological analysis indicated that the bivalent inactivated RHDV oil-emulsion vaccine was more effective than the bivalent inactivated RHDV aluminum hydroxide gel vaccine, resulting in improved immune responses and longer-lasting protective immunological responses in vaccinated rabbits. The bivalent inactivated RHDV oil-emulsion vaccine was also sterile and safe and helped the protection of the rabbits against RHDVa and RHDV2, hence reducing the time and effort required during the vaccination process and reducing the levels of discomfort for the rabbits.

**Keywords:** Immunity, Inactivated vaccine, Oil emulsion, Rabbit hemorrhagic disease virus

## INTRODUCTION

Rabbit hemorrhagic disease (RHD) is an acute, fatal viral disease that causes substantial losses in rabbit populations (Dalton et al., 2015). In Egypt, RHD was documented in adult rabbits in Assiut area during the winter of 1992 (Salem and El-Ballal., 1992). Rabbit hemorrhagic disease virus (RHDV) is a single-stranded, non-enveloped virus that belongs to the genus *Lagovirus* (family Calciviridae). The current nomenclature of this virus is based on several evolutionary relationships, with *Lagovirus europaeus* being a species of *Lagovirus*. Within this species, RHDV (G1) and European brown hare syndrome virus (G2) are divided into two genogroups: G1.1a/RHDVa for the G6 RHDVa strains, G1.1b/RHDV for the classical RHDV G1 strains, G1.1c/RHDV for the classical G2 strains, and G1.1d/RHDV for the classical G3, G4, and G5 strains. G1.2/RHDV2/b is a newly proposed label for the recently described RHDV2 (Le Pendu et al., 2017).

The RHDV2 was first discovered in 2010 in France (Le Gall-Reculé et al., 2011) and later discovered in a number of governorates in Lower Egypt in 2018 and 2019 (Erfan and Shalaby, 2020; Hamida et al., 2020). Generally, RHDVa is characterized by a high mortality rate (70-90%) with a subclinical form in rabbits aged less than 6-8 weeks, whereas RHDV2 is associated with a high mortality rate (50-70%) in young rabbits but a lower rate (20-30%) in adult rabbits (Puggioni et al., 2013).

The RHDV2 was discovered in 2018 and 2019 in several governorates in Egypt. To control the spread of RHD, rabbits are vaccinated with inactivated RHDV vaccines (OIE, 2018). However, several RHD outbreaks have been reported among rabbits vaccinated by commercially available vaccines containing classical or variant strains (RHDVa). Therefore, unique RHDV was antigenically isolated and called RHDV2 (Le Gall-Reculé et al., 2011; Dalton et al., 2012). However, no cross-protective effect was observed between RHDVa and RHDV2 (Bárcena et al., 2015; OIE, 2019; Abd El-Moaty et al., 2020). According to the World Organisation for Animal Health (OIE), rabbits should be vaccinated by vaccines containing two types of RHDV (OIE, 2019).

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Montanide incomplete seppic adjuvant (ISA) is an oil-emulsion emulsifier and immuno-modulator used to produce different oil-emulsion veterinary vaccines to improve the immune response (Suckow et al., 2012). The first goal of this study was to develop a bivalent inactivated RHDV oil-emulsion vaccine (Montanide ISA 71 VG) and compare it with a vaccine containing antigenic RHDVa and RHDV2 adjuvanted with an aluminum hydroxide gel. The other goal was to evaluate the efficacy and safety of this vaccine to help control RHD outbreaks in Egypt.

## MATERIALS AND METHODS

### Ethical approval

This study was approved by the Veterinary Serum and Vaccine Research Institute (VSVRI) Abbasia, Agriculture Research center, Ministry of Agriculture, Cairo, Egypt. All procedures and rabbit care steps were performed following the institutional rules for using animals in research.

### Rabbit hemorrhagic disease virus

Two Egyptian RHDV strains (Giza/2006 RHDVa strain and Mahala2019/VSVRI RHDV2 strain) were provided by the VSVRI for vaccine manufacture, challenge of vaccinated rabbits, and hemagglutination inhibition (HI) test.

#### *Rabbit hemorrhagic disease virus a*

Giza/2006 is a local Egyptian strain of RHDVa (accession no. HE963222) with a titer of  $10^{6.5}$  LD<sub>50</sub>/mL and a hemagglutination (HA) titer of  $2^{14}$  HA units.

#### *Rabbit hemorrhagic disease virus 2*

Mahala2019/VSVRI is a local Egyptian strain of RHDV2 (accession no. MK736667) with a titer of  $10^{6.7}$  LD<sub>50</sub>/mL and an HA titer of  $2^{12}$  HA units.

### Experimental rabbits

A total of 280 New Zealand susceptible male rabbits (1.5-2.0 kg) were obtained for preparation (10 rabbits, 8 weeks old), safety tests (20 rabbits, 8 weeks old), and vaccine evaluation (250 rabbits, 6 weeks old). All rabbits used were confirmed to be seronegative for RHDV through the HI test.

### Preparation of vaccines

All procedures were performed in compliance with the OIE Terrestrial Manual (OIE 2018) in the virology laboratory of VSVRI, Agricultural Research Center, Abbasia, Ministry of Agriculture, Cairo, Egypt. Briefly, the two viruses were first propagated in seronegative susceptible rabbits, and then the supernatants of RHDVa and RHDV2 were separately inactivated for 48 hours with formalin at a final concentration of 0.4% at 37°C. Viral inactivation was then determined by injecting five rabbits with an inactivated suspension and using two rabbits as a control group. The inactivated suspension was deemed ready for emulsification with a vaccine adjuvant if the infected rabbits demonstrated no clinical evidence of illness or fatality. Subsequently, a 2% aluminum hydroxide gel (constituting 20% of the vaccine volume) or Montanide ISA 71 VG oil emulsion was used as a solution adjuvant (constituting 70% of the preparation volume), Registered in General organization for veterinary services under registration number 899 with trade name Servac bivalent RHDV gel vaccine and number 855 with trade name Servac bivalent RHDV oil vaccine. Finally, the vaccine dose was modified to  $2^{10}$  HA units in 0.5 mL per rabbit and subcutaneously administered in single injections (Peshev and Christova, 2003; OIE, 2018; El-Jakee et al., 2019;).

### Sterility test

The two prepared vaccines were tested for sterility following standard cultivation procedures in aerobic and anaerobic bacterial and fungal growth media and then examined daily for 14 days (OIE, 2018).

### Safety

To test the safety of administering an overdose of the vaccine (three doses of the inactivated vaccine), 10 seronegative rabbits were each subcutaneously injected with 1.5 mL of the vaccine. Then, at 3 weeks post-vaccination (WPV), the rabbits were monitored for any signs of illness or local response (OIE, 2018).

### Experimental design

A total of 250 susceptible rabbits, aged 6 weeks, were housed in sterilized metal cages in a well-ventilated and disinfected open yard at a temperature of approximately 25°C and relative humidity of 50%. The rabbits were fed with commercial pellets (18% proteins and 14% fibers, with a total energy of 2550 kcal) purchased from Atmida (Cairo, Egypt) and allowed to drink water ad libitum. All rabbits were kept for 2 weeks before use in the experiment to be sure that they were seronegative to RHDV. The HI testing indicated that the rabbits were seronegative to both RHDV antibody strains.

Therefore, all rabbits were divided into three groups and each group had five replicates. Group 1 (100) were subcutaneously injected with the bivalent inactivated RHDV vaccine with an aluminum hydroxide gel at a dose of 0.5 mL per rabbit (single dose), and Group 2 (100) were subcutaneously injected with the bivalent inactivated RHDV oil-emulsion vaccine at a dose of 0.5 mL per rabbit (single dose). Group 3 (50) were subcutaneously injected with normal physiological saline at a dose of 0.5 mL per rabbit as a placebo and were left unvaccinated as the control group (Kim et al., 1989).

Each group of rabbits was individually housed and kept under daily observation until the experiment was complete. Individual rabbit serum samples were obtained for all groups starting on the first day, weekly after vaccination until 4 WPV, every two weeks until 8 WPV, and then monthly until 12 WPV. Individual rabbit sera were inactivated by boiling in a water bath at 56°C for 15 minutes and stored in sterile vials at -20°C until serological analysis for specific RHDV antibodies with an HI test (Kim et al., 1989; Yuan et al., 2013).

### Hemagglutination inhibition test

Each serum sample was tested for RHDVa and RHDV2 antigens twice. Before incubation at 37°C for 30 minutes, twofold serial dilutions of the serum samples were performed in 50 µL of phosphate-buffered saline, and an equal amount of virus antigen containing eight HA units were added. Then, 0.75% human red blood cells (type O) were added (50 µL) and incubated at 4°C for 1 hour. Finally, the serum dilution that demonstrated HA inhibition, as measured by mean HI log<sub>2</sub>/mL titers, was considered the endpoint (Peshev and Christova, 2003; OIE, 2018).

### Virus challenge

A total of 40 rabbits from vaccinated groups (20 rabbits from each vaccinated group) and 10 rabbits from the unvaccinated group were randomly chosen for vaccine evaluation at 3 WPV, 3 months post-vaccination (MPV), 6 MPV, and 12 MPV. Then, to conduct the challenge, rabbits selected from each vaccinated group were divided into two subgroups of 10 rabbits each. Each immunized group received two types of RHDV. All rabbits in the control group received an intramuscular injection containing 1 mL of a suspension with 10<sup>3</sup> LD<sub>50</sub> virulent RHDVa and 1 mL of a suspension with 10<sup>3</sup> LD<sub>50</sub> virulent RHDV2 (single injection). Subsequently, all rabbits were kept under constant observation for 2 weeks, and the numbers of mortalities and postmortem lesions were documented.

## RESULTS

In this study, an inactivated RHDV oil-emulsion vaccine was compared to an inactivated RHDV vaccine with an aluminum hydroxide gel. The HI tests and vaccine challenges were conducted to assess humoral immunity. Table 1 shows the estimated mean specific RHDV antibodies. Before vaccination, none of the vaccinated or unvaccinated control rabbits had RHDV particular antibodies. The humoral immune response was measured against two viruses (RHDVa and RHDV2) for each vaccine. At 1 WPV, the mean titers for specific anti-RHDV antibodies were, respectively, 2<sup>6</sup> and 2<sup>5.75</sup> in the gel vaccine group and 2<sup>4</sup> and 2<sup>4.2</sup> in the oil-emulsion vaccine group for RHDVa and RHDV2. The anti-RHDV antibody titers then gradually increased in the two groups, peaking at, respectively, 2<sup>11</sup> and 2<sup>11.5</sup> at 6 WPV in Group 1 and 2<sup>11.5</sup> and 2<sup>11.4</sup> at 5 MPV in Group 2 for RHDVa and RHDV2. The maximum mean RHDV antibody titer for RHDVa and RHDV2 was reached at 6 WPV for Group 1 and 5 MPV for Group 2.

In Group 1, the mean titers for the specific anti-RHDV antibodies for RHDVa and RHDV2 gradually increased at 1 WPV, reached their peak at 6 WPV, and then decreased but remained protective until 6 MPV; the mortality rate was 100% at 12 MPV. In Group 2, the mean titers for the specific anti-RHDV antibodies for RHDVa and RHDV2 gradually increased at 1 WPV, reached their peak at 5 MPV, and then decreased but remained protective until 12 MPV, and the mortality rate was 0% at 12 MPV.

The results of the challenge are shown in Table 2. In the current study, two local virulent strains (RHDVa and RHDV2) were used for the challenge of vaccinated rabbits with either the gel vaccine or the oil-emulsion vaccine as well as none vaccinated group at 4 intervals (3 WPV, 3 MPV, 6 MPV, 12 MPV).

These results indicated that the rabbits in both groups were completely protected against pathogenic RHDVa (10<sup>3</sup> LD<sub>50</sub>/mL) and RHDV2 (10<sup>3</sup> LD<sub>50</sub>/mL). As shown in Table 2, this protective effect was observed at 3 WPV, 3 MPV, and 6 MPV in Group 1 and remained until the end of the experiment (12 MPV) in Group 2.

The mortality rate was 100% at 12 MPV in the gel vaccine group, which could not resist the challenge by providing any protection. The mortality rate was 100% at 3 WPV, 3 MPV, 6 MPV, and 12 MPV in none vaccinated control group (3). The challenged rabbits of the control group could not resist the challenge by providing protection; where all rabbits died within 72 hours post challenge with specific and characteristic clinical signs and postmortem lesions of rabbit hemorrhagic disease virus and had no protection. The observed clinical signs were sudden death, nervous manifestations (ataxia, tremors, convulsions, and excitation), aimless running, lateral recumbence, paddling movement by legs, and crying before death. The characteristics of PM lesions of freshly dead rabbits were congestion and hemorrhages in the internal organs with hepatic necrosis and splenomegaly.

**Table 1.** Geometric means of rabbit hemorrhagic disease virus-specific antibody titers ( $\log_2$ ) in the sera of vaccinated and unvaccinated rabbits

Post-vaccination period	Geometric means of RHDV HI antibody titers ( $\log_2$ )					
	Group 1		Group 2		Group 3	
	RHDVa	RHDV2	RHDVa	RHDV2	RHDVa	RHDV2
Day 1	0	0	0	0	0	0
1 WPV	6	5.75	4	4.2	2*	1*
2 WPV	7.6	8	4	4.2	1	0
3 WPV	8.9	8	5.4	5	1	1
4 WPV	10.7	10.3	6.5	6	2	1
6 WPV	11	11.5	7	7.2	0	0
2 MPV	10.2	11	8	8.9	1	0
3 MPV	8.2	9	10.5	10	1	1
4 MPV	7	7.5	10.5	10	0	1
5 MPV	6.2	7	11.5	11.4	1	1
6 MPV	5	5.8	10.5	11	2	0
7 MPV	3.8	4	10	10.5	2	1
8 MPV	3.2	3.8	9	10	1	1
9 MPV	3	3	8.5	9.7	0	0
10 MPV	2.8	3	8	9	1	0
11 MPV	2.4	2.8	8	7.5	2	0
12 MPV	2	2.4	7	6.5	1	0

RHDV: Rabbit hemorrhagic disease virus, HI: Hemagglutination inhibition. RHDVa: Rabbits challenged with virulent RHDVa, RHDV2: Rabbits challenged with virulent RHDV2, WPV: Weeks post-vaccination, MPV: Months post-vaccination. Group 1 included rabbits that received the gel vaccine, Group 2 included rabbits that received the oil-emulsion vaccine, and Group 3 included the unvaccinated rabbits. \*Antibody titers were non-specific and non-protective. \*Protection value is considered above  $2^4$ .

**Table 2.** Potency of the bivalent inactivated rabbit hemorrhagic disease virus oil-emulsion vaccine with a Montanide adjuvant and an aluminum hydroxide gel adjuvant

Post-vaccination period	Group	Challenge virus	Number of challenged rabbits	Number of protected rabbits	Number of dead rabbits	Protection level (%)
3 WPV	1	RHDVa	10	10	0	100
		RHDV2	10	10	0	100
	2	RHDVa	10	10	0	100
		RHDV2	10	10	0	100
	3	RHDVa	5	0	5	0
		RHDV2	5	0	5	0
3 MPV	1	RHDVa	10	10	0	100
		RHDV2	10	10	0	100
	2	RHDVa	10	10	0	100
		RHDV2	10	10	0	100
	3	RHDVa	5	0	5	0
		RHDV2	5	0	5	0
6 MPV	1	RHDVa	10	10	0	100
		RHDV2	10	10	0	100
	2	RHDVa	10	10	0	100
		RHDV2	10	10	0	100
	3	RHDVa	5	0	5	0
		RHDV2	5	0	5	0
12 MPV	1	RHDVa	10	0	10	0
		RHDV2	10	0	10	0
	2	RHDVa	10	10	0	100
		RHDV2	10	10	0	100
	3	RHDVa	5	0	5	0
		RHDV2	5	0	5	0

RHDV: Rabbit hemorrhagic disease virus, RHDVa: Rabbits challenged with virulent RHDVa, RHDV2: Rabbits challenged with virulent RHDV2, WPV: weeks post-vaccination, MPV: Months post-vaccination. Group 1 included rabbits that received the gel vaccine, Group 2 included rabbits that received the oil-emulsion vaccine, and Group 3 had the unvaccinated rabbits.

## DISCUSSION

Rabbit hemorrhagic disease is rabbits' most common viral disease, which is associated with a high mortality rate and substantial economic losses (Dalton et al., 2015). All RHD isolates were believed to be antigenically linked until 1997, when (Capucci et al. 1998) discovered a variant with different genetic and antigenic properties, which they called RHDVa. This variant was found and isolated in Egypt in 2006 (Salman, 2007). In 2018 and 2019, another variant called RHDV2 was detected in certain Egyptian governorates, associated with substantially high mortality rates, particularly in young rabbits (Abido et al., 2020; Erfan and Shalaby, 2020). In 2019, several RHDVa variant strains were detected and verified in multiple regions in Upper Egypt, posing a threat to the population of rabbits (Abodalal et al., 2021). These findings agree with those of Mahar et al. (2018), who detected both circulating RHDVa and RHDV2 strains. Abd El-Moaty et al. (2020) reported both conventional (G1.1d/RHDV) and variant (G1.1a/RHDVa) genotypes co-exist in Egyptian rabbit populations.

Generally, RHD control can be achieved using inactivated RHDV tissue vaccines (Abido et al., 2020). Limited immunological cross-protection exists between genotypes G1.1a and G1.2 (Calvete et al., 2018). Although cross-protection immunity has been observed between classical and variant RHDVa (Read and Kirkland, 2017; Abd El-Moaty et al., 2020), both RHDVa and RHDV2 exhibit no cross-protection immunity (OIE, 2018). Therefore, a multivalent RHDV vaccine should be considered because of the limited cross-protection observed in rabbits that have received monovalent vaccines (Connor et al., 2022).

In the present study, humoral immune responses were assessed against both RHDVa and RHDV2. In previous studies, humoral immune responses were assessed against either RHDVa or RHDV2 (Montbrau et al., 2016; Abido et al., 2020) because only one monovalent RHDV vaccine was available.

According to Salman (2007), the protective value of an antibody titer is  $2^4$ , below which the titer is considered to be a non-specific, non-protective titer. In the present study, all vaccinated rabbits demonstrated protective serum antibody responses with detectable antibody titers, whereas the unvaccinated rabbits demonstrated no detectable RHDV antibody responses (Table 1). According to the OIE (2018), inactivated adjuvant vaccines induce robust protective immunity against RHD infection during 7-10 days.

Specific anti-RHDV HI antibodies against RHDVa and RHDV2 were detected at 1 WPV, which accords with the results of Smid et al. (1991), who reported the presence of anti-RHDV HI antibodies against RHDVa at 1 WPV. The inactivated RHDV gel vaccine elicited rapid immunity in the vaccinated rabbits, as evidenced by the mean titers of specific RHDV HI antibodies at 1 WPV ranging from  $2^6$  to  $2^{5.75}$  for RHDVa and RHDV2, respectively, in Group 1. The same results were obtained by Abodalal and Tahoon (2020). These results agree with those of Abido et al. (2020), who reported a value of  $2^6$  for the RHDV2 gel vaccine, but a lower value than that obtained by Salman (2007) ( $2^{8.2}$ ) for the RHDVa gel vaccine.

Comparison of the results obtained for Group 2, which received an oil-emulsion vaccine, with the results obtained with other oil-emulsion vaccines indicated that the specific titer for vaccinated rabbits is  $2^4$  for RHDVa at 1 WPV, which is lower than  $2^{7.7}$ , and  $2^5$  that were reported by El-Maghraby et al. (2019). Such an increased antibody titer following vaccination with an oil-emulsion vaccine can be attributed to its low viscosity and high homogeneity (Gomes et al., 1980). These results agree with those of Peshev and Christova (2003), who also used an RHDV oil adjuvanted vaccine and detected HI antibodies ( $2^{6.12}$ ) at 1 WPV.

At 1 WPV, the specific antibody titer for RHDV2 was found to be  $2^{4.2}$ , which is lower than the value obtained by Abido et al. (2020) ( $2^{6.7}$ ). This discrepancy was because Abido et al. (2020) used a different type of adjuvant (i.e., Montanide ISA 206). These results align with those of Montbrau et al. (2016). They reported that simultaneous administration of inactivated oil classical RHDVa and inactivated oil variant RHDV2 vaccines improves immunity levels starting at 7 days after vaccination.

In Group 1, the RHDV HI antibody mean titers gradually increased, reaching  $2^{8.9}$  and  $2^8$  for RHDVa and RHDV2, respectively, at 3 WPV. These results agree with those of Abodalal and Tahoon (2020) (who reported higher titers of  $2^{10.7}$  and  $2^{10.3}$ ) and Abido et al. (2020) (who also reported a higher RHDV2 HI antibody titer of  $2^{8.9}$ ). As evidenced by the elevated HI titers, these concentrations stimulated a strong humoral immune response against RHDVa and RHDV2, peaking at  $2^{11}$  and  $2^{11.5}$  for RHDVa and RHDV2, respectively, at 6 WPV. However, these values decreased at 2, 3, 4, 5, and 6 MPV but remained protective until finally decreasing again at 7 MPV. These results agree with those of Abodalal and Tahoon (2020), who reported that the RHDV antibody levels gradually increased and then decreased. In the present study, the mean RHDV antibody titers in Group 1 first increased and then decreased (bell shape). However, in Group 2, the mean RHDV antibody titers gradually increased, reaching 211 and 211.5 for RHDVa and RHDV2, respectively, at 5 MPV, then gradually decreasing but remaining protective, at  $2^7$  and  $2^{6.5}$  for RHDVa and RHDV2, respectively, at 12 MPV. These results are in line with those obtained by EL-Maghraby et al. (2019).

The results obtained in this study showed that the bivalent oil-emulsion vaccine maintained the antibody titers for a longer period of time, resulting in antibody titers of  $2^7$  and  $2^{6.5}$  for RHDVa and RHDV2, respectively, at 12 MPV. These

values are better than those obtained with the aluminum hydroxide gel vaccine, which may be attributed to the slow release of the oil adjuvant vaccine antigen. These results agree with those of Huang (1991), who reported that the oil-emulsion vaccine induced higher and longer-lasting antibody titers than those obtained with the RHDV aluminum hydroxide gel vaccine. In conclusion, the oil-emulsion vaccine provided a slow but long-term (1 year) immune response, whereas the gel-based vaccine (water-based) provided a fast but shorter-term (6 months) immune response.

As shown in Table 2, challenging virulent RHDVa and RHDV2 ( $10^3$  LD<sub>50</sub>/mL) resulted in 100% protection against both viruses at 3 WPV. This effect lasted until 6 MPV in Group 1 and until the end of the experiment (12 MPV) in Group 2. Mortality was observed in 100% of the unvaccinated rabbits and the vaccinated rabbits of Group 1 at 12 MPV within 72 hours. No mortality was observed in Group 2. The rabbits demonstrated no sign of illness at 12 MPV, indicating that the oil-emulsion vaccine provided 100% protection against clinical signs and mortality.

This protective effect was observed at 3 WPV and lasted until 12 MPV. These findings agree with those of Shevchenko (1994), who reported 100% protection in vaccinated rabbits challenged with virulent RHDVa at 12 MPV, and Montbrau et al. (2016), who also reported 100% protection in vaccinated rabbits challenged with a virulent RHDV2 strain 7 days after vaccination.

The results further indicated that the oil-emulsion vaccine had an adequate concentration of antigens, which helped induce a high antibody titer and provided 100% protection. These results are in line with those of Stone et al. (1983) and are also consistent with those of Salman (2007), who reported that adult rabbits with RHDV antibody titers ranging from  $2^6$  to  $2^{13}$  remained clinically healthy after being inoculated with a virulent RHDV.

## CONCLUSION

Inactivated RHDV oil-emulsion vaccines are superior to aluminum hydroxide gel vaccines in providing long-term immunity. They can be safely used for the active immunization of rabbits against RHDV, which is considered a threat to the Egyptian rabbit industry. The vaccination process can first be initiated with a bivalent aluminum hydroxide gel vaccine to obtain a fast response and then with a bivalent oil-emulsion vaccine to achieve long-term immunity.

## DECLARATIONS

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### Authors' contributions

Samah El Sayed Abodalal designed the study and prepared the vaccination batches. Samah El Sayed Abodalal and Mohamed Abdelkhalek Abdrabo performed the experimental and serological procedures. Samah El Sayed Abodalal analyzed and interpreted the data and wrote the manuscript. This manuscript content was authored, reviewed, and approved by Lamiaa Mohamed Omar, Mohamed Abdelkhalek Abdrabo, and Samah El Sayed Abodalal for publication.

### Competing interests

The authors declare no competing financial or personal interests that may have influenced the research presented herein.

### Ethical considerations

All authors approved the final version of this manuscript for publication. They also confirm that this study is free from any ethical concerns, such as plagiarism, consent to publish, misconduct, data fabrication and/or falsification, double publishing and/or submission, and redundancy. All data related to this study are prepared for publication in the present journal.

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