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Comparative efficacy of locally prepared inactivated *Rabbit Haemorrhagic Disease Virus* vaccines with available commercial vaccines

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ABSTRACT

Rabbit haemorrhagic disease (RHD) is an acute infectious disease that affects young and adult rabbits with high mortality rates, causing damage to the rabbit industry. Currently, vaccination is the main modality for controlling the rabbit haemorrhagic disease virus (RHDV). The purpose of the current investigation was to prepare and evaluate the protective efficacy of a homologous vaccine against RHDVa and RHDV2 strains and compare them with available commercial vaccines, in addition to determining the cross-protection between the two isolated strains (RHDVa and RHDV2). The inactivated RHDV vaccines were prepared using montanide ISA-71 oil adjuvants. The prepared vaccines were sterile and safe. Sero-negative rabbits were vaccinated at two months with locally prepared RHDVa and RHDV2, as well as compared with imported RHDVa and RHDV2 vaccines. The rabbits were challenged at 3rd-week post-vaccination with local RHDVa and RHDV2 strains. The result revealed that the homologue vaccine strain achieved a significant level of protection but no significant difference between the local and imported vaccines. In addition, there is no cross-protection between two locally isolated strains.

1. INTRODUCTION

Rabbit haemorrhagic disease (RHD) is a highly contagious and lethal haemorrhagic disease in rabbits. RHDV is a species-specific lagovirus that belongs to the Caliciviridae family and has a single-stranded, positive-sense RNA genome (Buehler et al., 2020). Biosecurity approaches for the prevention and control of RHD, including monitoring, sanitation, disinfection, and quarantine, are highly important to limit or prevent the disease in the rabbit industry. In addition, these measures might prevent widespread infection in countries where RHDV circulates in wild rabbits and where eradication is not available (Abrantes et al., 2012). In addition to vaccination with a proper vaccine, a further RHDV control strategy. For the last 20 years, successful RHDV control has been simple due to the adoption of an efficient vaccination and the low antigenic diversity of field virus strains (Lavazza and Capucci, 2012). Firstly, the Egyptian classical strain was used for the preparation of inactivated RHDV formalized vaccines in Egypt (Daoud et al., 1998); following that, in 2006, RHDVa variant strains were identified and began to replace the classic RHDV strain in the manufacture of the vaccine in 2008 (Abodalal and Tahoon et al., 2020). RHD outbreaks with high mortality were detected in several flocks of rabbits that were vaccinated with available commercial vaccines manufactured from classic or variant strains of RHDV (RHDV/RHDVa). This is due to the emergency of RHDV2, which is a new RHDV strain that is antigenically distinct from the classical strain (Dalton et al., 2012; Le Gall-Reculé et al., 2013; Hemida et al., 2020). There is some cross-protection immunity between classical and variant RHDVa strains (Read and Kirkland, 2017; Abd El-Moaty et al., 2020), but not much. There is also some cross-protection immunity between RHDVa and RHDV2 (Bárcena et al., 2015; Connor et al., 2022). It was recommended to vaccinate rabbits with a vaccine containing the homologous strain to that detected during the outbreak or contain both antigenic types (RHDVa and RHDV2) (OIE, 2021). In Egypt, commercially accessible RHD vaccines such as the bivalent Servac® RHDV and Curnipravac® vaccine are the backbone of the present RHDV management approach

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(Salman, 2007). The purpose of the current study was to prepare and evaluate the protective efficacy of the homologous vaccine against local RHDVa and RHDV2 strains and compare them with available commercial vaccines containing the same strains, in addition to determining the cross-protection between RHDVa and RHDV2.

2. MATERIAL AND METHODS

2.1. Ethical Approval

The Institutional Animals Care and Use Committee, Research Ethics Board, Faculty of Veterinary Medicine, Benha University (No. BUFVTM 331022) approved the study protocols, following animal welfare guidelines.

2.2. Experimental rabbits

In our study, a total of 232 seronegative New Zealand white rabbits of 7 weeks of age and weighting about 1.5–2 kg were used. The rabbits were confirmed to be seronegative for both RHDV strains through the HI test. These rabbits were required for the determination of lethal dose 50 (LD50) (60 rabbits), preparation (12 rabbits), and evaluation of the vaccines (160 rabbits). They were raised and placed in stainless steel cages and monitored for a one-week acclimatization period.

2.3. Local RHDV strains and commercial vaccines

Two locally isolated RHDV strains (RHDV-EGY-Qalubia-AS-5-2021 and RHDV-EGY-Assuit-AS-17-2022) were identified through haemagglutination tests (HA) and RT-PCR in addition to sequence and phylogenetic analysis (Desouky et al., 2023). RHDV-EGY-Assuit-AS 17-2022 (RHVa, accession number OP554373) has a HA titer of 211 and an infectivity titer of 10 6 LD50/mL, while RHDV-EGY-Qalubia-AS 5-2021 (RHDVb, accession number ON920552) has a HA titer of 212 HA units and an infectivity titer of 10 6.5 LD50/mL.

Two commercial vaccines were used, the inactivated "ERAVAC®" vaccine against RHDV2 (Batch No. AN: 01737/2020) and the inactivated "CUNIPRAVAC® (Batch No. 88B7-1/8.1) vaccine against RHDV1, with a recommended dose of 0.5 ml by subcutaneous route. The two vaccines were produced by the HIPRA company. These commercial vaccines were purchased from the sector.

2.4. RHDV propagation and determination of LD50

Sixty susceptible rabbits were used for propagation and calculation of the LD50 for RHDVa and RHDV2 viruses (30 rabbits /virus). This test was carried out according to OIE, 2021.

2.5. Inactivation of local RHDV strains

Inactivation of RHDVa and RHDV2 strains, according to OIE, 2021. Briefly, the supernatants of the RHDVa and RHDV2 isolates were collected and inactivated separately using formalin for 48 hours at a final concentration of 0.4% at 37 °C. The fluid was continually mixed during inactivation. The virus inactivation assessment was performed by injecting each inactivated suspension into five rabbits and keeping two rabbits as controls. These rabbits were kept under observation for two weeks.

2.6. RHDV vaccine preparation and evaluation

Preparation of inactivated RHDVa and RHDV2 vaccines according to OIE, 2021. The inactivated suspensions of two isolates were deemed ready for emulsification with the vaccine adjuvant if the injected rabbits showed no further clinical indications of the disease or mortality. The two suspensions were separately adjuvanted with Montanide ISA 71 oil (to occupy 70% of the preparation volume). This Montanide ISA 71 oil (Chem Trade Berkeley Heights, New Jersey) was applied in accordance with the manufacturer's recommendations. The appropriate vaccine dose (0.5 mL/rabbit) contained 210 HAU per vaccine dose and was administered through the subcutaneous (S/C) route (OIE, 2021). The prepared vaccines were tested for sterility, safety, and efficacy in accordance with OIE, 2021. The vaccines were examined for sterility or the absence of viable bacteria and fungi. This was achieved by culture on media such as nutrient and sabaroud agar, while safety was applied through the inoculation of 10 seronegative rabbits (5 rabbits/ vaccine) with a double vaccine dose by the S/C route. These rabbits were monitored for 3 weeks' post-inoculation. Finally, the assessment of the vaccine efficacy depended on the immune response, which was determined through the HI test and challenge test.

2.7. Experimental design for comparing local with commercial RHDV vaccines

As shown in Table 1, one hundred and fifty rabbits (150) were equally divided into five groups (30 rabbits/group). The first group (1st group) was vaccinated at 2 months of age S/C with 0.5 mL locally prepared RHDVa vaccine; the second group (2nd group) was vaccinated at the same age with 0.5 mL imported RHDVa vaccine; the third group (3rd group) was vaccinated with locally prepared RHDV2 vaccine; and the fourth group (4th group) was vaccinated with imported RHDV2. Finally, the fifth group (5th group) was kept as a control group. Blood samples were collected before vaccination and then weekly after vaccination for three successive weeks. Twenty rabbits from groups 1 and 3 (20 rabbits/group) and ten rabbits from groups 2 and 4 were separated into separate rabbitries for the challenge at the 3rd

week post-vaccination. The rabbits were challenged intramuscularly with 1 mL of a suspension with 103 LD50 of isolated RHDVa and RHDV2 strains. The control group was divided into 3 subgroups, one of which was challenged with RHDVa and another with the RHDV2 strain, while the last remained as the control negative group (Table 1).

Table 1. Design for comparative efficacy of locally prepared and imported RHDV vaccines in rabbits

Group (30rabbit/group)		Vaccine type	Challenge virus (10 rabbit/virus)
Group 1		Locally prepared RHDVa	Locally isolated RHDVa Locally isolated RHDV2
Group 2		Imported RHDVa	Locally isolated RHDVa
Group 3		Locally prepared RHDV2	Locally isolated RHDVa Locally isolated RHDV2
Group 4		Imported RHDV2	Locally isolated RHDV2
Group 5	Positive Control	Non vaccinated challenge	Locally isolated RHDVa Locally isolated RHDV2
	Negative Control	Non vaccinated non challenge	Non challenge (control negative group)

Vaccination time: at 2 months of age, Route and dose: S/C with 0.5 mL of vaccine, Challenge time: at 3rd WPV

Clinical signs, mortality and lesions were monitored for 2 weeks post challenge (WPC). Blood samples were collected from survived rabbits at 1st and 2ndWPC

2.8. Haemagglutination (HA) test

In accordance with OIE, 2021 A two fold dilution of the RHDV with an equivalent amount of 0.75% concentration washed human RBCs type "O" was incubated at 4°C in a sealed U shaped-bottom micro-titer plate to measure 8 HAU used in HI test

2.9. Haemagglutination inhibition (HI) test

It was performed to determine specific RHDV antibodies in rabbit serum. Reference RHDV antibodies were generously provided by Veterinary Serum and Vaccine Research Institute. Briefly, twofold serial dilutions of the serum samples were performed in 50 µL of phosphate-buffered saline, add an equal volume of virus antigen containing eight HA units then, 50 µL of 0.75% human RBCs type "O" were added and incubated for 1 hour at 4°C .Finally, the serum dilution that demonstrated HA inhibition, as measured by mean HI log2/mL titers, was considered the endpoint (OIE, 2021)

2.10. Statistical analysis

Significant differences between groups were determined by Mixed Way ANOVA followed by Tukey post hoc Test for pair wise comparison using IBM SPSS Statistics for Windows, Version 25.0.

3. RESULTS

3.1. Titration and inactivation of RHDV strains

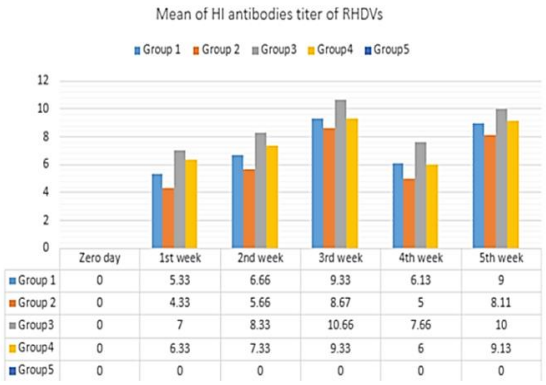
The titer of RHDVa strain was 106LD50/mL and a HA titer of 211 HA units, while RHDV2 strain titer was with 106.5 LD50/mL and an HA titer of 212 HA units. All rabbits injected with inactivated virus were kept alive without showing clinical sign

3.2. Sterility and safety of locally prepared inactivated RHDV vaccines

The sterility test revealed that the manufactured homologous vaccines had not been contaminated by bacteria or fungi. The prepared vaccines passed a safety test and were deemed to be safe. The inoculated seronegative susceptible rabbits with double field dose showed no clinical symptoms in the three weeks post vaccination.

3.3. Immune response of locally prepared and imported RHDV vaccines

Serum samples were collected for 5 weeks from all groups, three WPV and two WPC. Before vaccination, all groups had no RHDV antibodies titer which confirmed by HI test. The result revealed that all vaccinated groups demonstrated protective antibodies titer which measured through HI test. The specific anti-RHDV antibodies in all vaccinated groups appeared at 1stWPV then occur gradually and significant increase of antibodies titer to reach to high level at 3rdWPV , after the challenge occur significant decrease of antibodies titer at 1st WPC and began to increase secondly at 2ndWPC.In addition to the result demonstrated that the locally prepared monovalent inactivated RHDV vaccines (RHDVa or RHDV2 vaccine) induces similar immune response to the commercial imported vaccines in challenge with local isolates of the virus , there is no significant difference between them as shown in chart figure 1 and table 2. As shown in table 3, the protection rate at groups vaccinated with locally prepared and imported RHDVa vaccines was 90% while it ranged from 90-100% in rabbits vaccinated with imported and locally prepared RHDV2 vaccines respectively. In contrast in unvaccinated challenge group (control positive group) the mortality rates were 100% within 3 days of challenge with typical signs and lesions for RHD. The observed clinical signs were sudden death, bloody nasal discharge, paddling movement by legs, and finally appear nervous manifestations (ataxia, tremors and convulsions). The characteristics PM lesions of freshly dead rabbits were haemorrhages in lung, trachea and kidney with hepatic necrosis and splenomegaly, and some rabbits show haemorrhage at wall of stomach and over full urinary bladder as shown in figures 2, and 3.While unvaccinated unchallenged group (control negative group) showed no antibodies or immune response in addition to no clinical signs or mortality in this group. On the other hand, there is no cross protection between two locally isolated strains in challenge with heterologus strain, the mortality rate reach 100% within 5 days of challenge with typical signs and lesions for RHD.



	Zero day	1st week	2nd week	3rd week	4th week	5th week
Group 1	0	5.33	6.66	9.33	6.13	9
Group 2	0	4.33	5.66	8.67	5	8.11
Group3	0	7	8.33	10.66	7.66	10
Group4	0	6.33	7.33	9.33	6	9.13
Group5	0	0	0	0	0	0

Fig.1.Means of HI antibody titers (log2) in the sera of vaccinated and unvaccinated rabbits

Table 2.The mean of HI antibody titers Log_2 ± SE in the sera of vaccinated and unvaccinated rabbits

	Weeks post vaccination			Weeks post challenge		P value	
Groups	1st week	2nd week	3rd week	1th week	2nd week		
Group 1	5.33^{b}±0.33	6.66^{b}±0.33	9.33^{a} ±0.58	6.13^{b} ±0.33	9^{a} ±0.33	0.000	
Group 2	4.33^{b} ±0.58	5.66^{b}±0.33	8.67^{a}±0.33	5^{b} ±0.33	8.11^{a}±0.33	0.000	0.93
Group3	7^{b}±0.57	8.33^{b} ±0.57	10.66^{a} ±0.00	7.66^{b} ±0.33	10^{a} ±0.57	0.000	0.84
Group4	6.33^{b}±0.33	7.33^{b}±0.57	9.33^{a} ±0.57	6^{b} ±0.33	9.13^{a} ±0.33	0.000	
Group5	0±0	0±0	0±0	0±0	0±0	0±0	

**No detectable antibodies titer at zero day

Table 3. Protection and Mortality percent in rabbits vaccinated by local and imported RHDVa and RHDV2vaccines

Group No.	Vaccine type	Challenge virus	No of challenged rabbits	No of dead Rabbits	Mortality %	Survived	Protection%
Group1	Local RHDVa	Locally isolated RHDVa	10	1/10	10%	9/10	90%
		Locally isolated RHDV2	10	10/10	100%	0/10	0%
Group2	Local RHDVa	Locally isolated RHDVa	10	1/10	10%	9/10	90%
Group3	Local RHDV2	Locally isolated RHDVa	10	10/10	100%	0/10	0%
		Locally isolated RHDV2	10	0/10	0%	10/10	100%
Group4	Local RHDV2	Locally isolated RHDV2	10	1/10	10%	9/10	90%

Rabbits in control positive groups showed no protection with 100% mortality while the control negative group showed no mortality with 100% protection

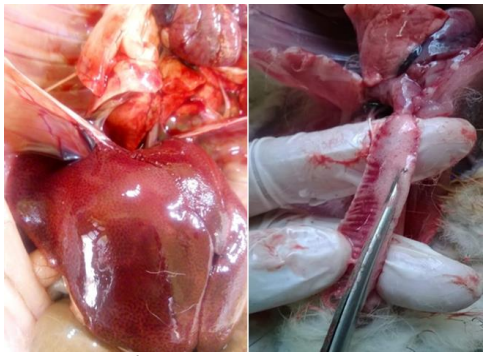


Fig.2 Post mortem lesions of RHD in control positive groups showing hepatic necrosis and haemorrhage in lung (right picture) and Frothy blood presents in trachea of rabbits (left picture)

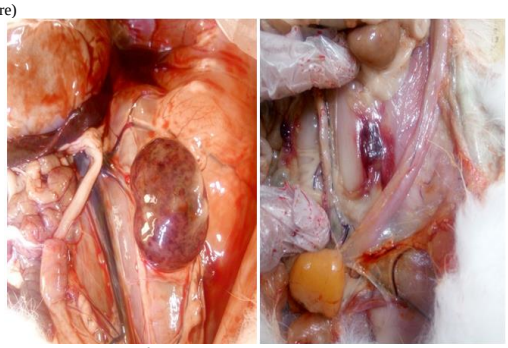


Fig.3 Post mortem lesions of RHD showing sever renal hemorrhage in RHDV2 challenged group (right picture) and impacted urinary bladder with discolored urine in positive control group (left picture)

4. DISCUSSION

Rabbit haemorrhagic disease is the most prevalent disease, characterized by a high mortality rate that threatens the rabbit population (Dalton et al., 2015). There are different genotypes of RHDV circulating in different Egyptian governorates, the variant strain RHDVa was identified in Egypt in 2006 (Salman, 2007). RHDV2, another variant, was identified in some Egyptian governorates in 2018 and 2019, characterized by high fatality rates, especially in young rabbits, in addition to substantial economic losses in the rabbit industry (Abido et al., 2020; Erfan and Shalaby, 2020). Vaccination is the most effective strategy to decrease the wide spread of the disease (Salman, 2007; Abido et al., 2020). In the present study, the locally prepared monovalent inactivated RHDV vaccines induce the same immune response as commercially imported vaccines; there is no significant difference between them. This finding disagrees with Soliman et al. (2020) finding that the locally manufactured monovalent inactivated RHDV vaccine with an aluminum hydroxide gel adjuvant induced a better immune response than the imported vaccine when the rabbits were challenged with local virulent isolates of the virus. This difference may be related to the preparation of the monovalent inactivated RHDV vaccine with a different

adjuvant (an aluminum hydroxide gel). Inactivated adjuvant vaccines elicit a protective immune response against RHD infection 7–10 days' post-vaccination (OIE, 2018).

An antibody's protective titer is 24 using HI test, below which the titer is regarded as a non-protective titer (Salman, 2007). In the current investigation, all vaccinated rabbits showed a protective and detectable titer of antibodies beginning with 1st WPV, while unvaccinated rabbits revealed no detectable RHDV immune responses, as shown in Table 2. At 1st WPV, specific antibodies were found against the RHDVa and RHDV2 vaccines, which is consistent with the findings of Smid et al. (1991), who noted the presence of specific antibodies against RHDVa at 1st WPV.RHDVa inactivated vaccines induce a protective and rapid immune response in the vaccinated rabbits, as the mean titers at the 1st WPV range from 24.33 to 25.33 for imported RHDVa and locally prepared RHDVa vaccines, respectively. This result is in agreement with El-Maghraby et al. (2019), who reported that the mean titer for vaccinated rabbits is 25 for RHDVa at 1st WPV. Also, this finding is nearly conceded by Abodalal et al. (2022) and Abodalal and Tahoon (2020), who demonstrated that the RHDV vaccine at 1st WPV induces rapid immunity in the immunized rabbits, with the mean titers of RHDV antibodies ranging from 26 for RHDVa.

RHDV2 inactivated vaccines also induce a protective and rapid immune response in the vaccinated rabbits, as the mean titers at 1st WPV 26.33 for imported RHDV2 vaccine and 27 for locally prepared RHDV2 vaccine. These findings are in line with those of Abido et al. (2020), who show that the titer 26 for the RHDV2 vaccine. Additionally, this result is nearly consistent with Abodalal et al. (2022), who demonstrated that the RHDV vaccine at 1stWPV induces mean titers of RHDV antibodies 2 5.75 for RHDV2. The mean antibody titers for RHDV gradually and significantly increased, reaching 28.6 for imported RHDVa vaccine and 29.3 for locally prepared vaccine and 2 9.3-2 10.6imported and locally prepared RHDV2vaccine respectively, at 3rdWPV, this result coincided with Abodalal et al., 2022 the RHDV HI antibody titers at 3rdWPV reached to 2 8.9 for RHDVa and 28for RHDV2.The protection rate at vaccinated groups 90% for RHDVa and 90-100% for RHDVbvaccine.While unvaccinated challenge group (control positive group) the mortality rate reached 100% within 3 days of the challenge, with typical signs and lesions for RHD. The observed clinical signs were sudden death, bloody nasal discharge, paddling movement by legs, and finally nervous manifestations (ataxia, tremors, and convulsions). The characteristics of PM lesions in freshly dead rabbits were haemorrhages in the lung, trachea, and kidney with hepatic necrosis and splenomegaly, and some rabbits showed haemorrhages at the wall of the stomach and overflowing urinary bladder. This result is in accordance with Abodalal et al. (2022), who reported that the vaccinated group's protection rate reached 100% and the challenged rabbits of the control group had no resistance and died within 72 hours' post-challenge with distinct clinical symptoms and postmortem lesions of rabbit haemorrhagic disease virus. The noticed clinical signs include sudden death, nervous signs (convulsions, ataxia, tremors, and excitation), aimless running, lateral recumbence, paddling movement by legs, and crying before death, while PM lesions were congestion and haemorrhages in the internal organs with liver necrosis and splenomegaly. On the other side, RHDVa and RHDV2 do not show cross-protection between each other. This result was conceded by Bárcena et al. (2015), who recorded that there is no cross-protection immunity between RHDVa and RHDV2.

5. CONCLUSIONS

The vaccination strategy is crucial for RHD control in the rabbit sector, and the results presented here demonstrate that the locally prepared vaccines achieved a significant level of protection and that there is no significant difference in immune response between locally prepared vaccines and imported ones. As there is no cross-protection between RHDVa and RHDV2, it is recommended to vaccinate rabbits with a vaccine containing the same strain that was detected during the outbreak or a bivalent vaccine containing both antigenic types (RHDVa and RHDV2) to control both RHD virus outbreaks in Egypt and minimize economic losses.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest for current data

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