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Preparation of Locally Prepared Inactivated Combined Vaccine of Rabbit Hemorrhagic Disease Virus Types 1 & 2 and *Pasteurella multocida*

Samah El Sayed Ali Abodalal[1*] and Mahmoud T.A. Ismail[2]

[1]Newcastle Disease Research Department; [2]Aerobic Bacteria Research Department, Veterinary Serum and Vaccine Research Institute (VSVRI), Abbasia, Agricultural Research Center (ARC), Cairo, Egypt
***Corresponding author:** drsamahsaidvet@gmail.com

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ABSTRACT

A combined inactivated montanide ISA-70 adjuvanted vaccine against two types of rabbit viral hemorrhagic disease (RVHD) and Pasteurella multocida (P. multocida) was prepared. Another 2 inactivated vaccines were prepared too, one bivalent RHDV1 and RHDV2 against RVHD while the other single monovalent against P. multocida. The prepared vaccines were compared from the aspect of immune response up on the vaccinated rabbits. The comparison was based on the estimation of the humoral immune response for both RHDV and *P. multocida* by hemagglutination inhibition test (HI) and indirect hemagglutination test (IHA). Four groups of susceptible rabbits were used; one for each vaccine and the 4th group was control. The immune response was followed up for 24 weeks. Higher and earlier immune response against both RHDV1and RHDV2 were induced by combined oil emulsified vaccines at 1st week post vaccination (WPV) than bivalent inactivated RHDV vaccine. Using HI, the antibody titer reached maximum levels of combined oil vaccine (211.75, 211.5) for RHDV1 and RHDV 2 at the 12th weeks after vaccination, respectively. The same results were for P. multocida vaccine as the higher and earlier immune response against P. multocida was obtained from the combined vaccine than Pasteurella vaccine only. For P. multocida A and D, at the 6th week after vaccination, antibody titers of combined oil vaccine using IHA reached (218.5,218.2) respectively. The challenge test in the combined vaccine group revealed that, RHDV1 gave protection with 90%, and 100% for RHDV2 in the combined vaccine. As well as challenge test for Pasteurella gave protection with 90%. The developed combined inactivated montanide oil vaccines against Rabbit hemorrhagic disease types 1, 2 and Pasteurellosis appeared to be safe and effective in improving the immune response (onset and longevity). Immune protection against RHDV1, RHDV2 and P. multocida can be achieved with one manipulation decreasing the stress on animals and efforts.

Key words: Combined, RHDV, Pasteurella, Vaccine, HI, Rabbits.

INTRODUCTION

Rabbit hemorrhagic disease virus (RHDV) is a highly contagious and acute viral disease that affects rabbits worldwide resulting in 90% mortality rates in both wild and domestic rabbits. RHDV is one of the members of the genus Lagovirus under Caliciviridae family. The virus is non-enveloped, icosahedral, single strand, positive sense RNA virus (Magouz et al. 2019; Abd El-Moaty et al. 2020; Soliman et al. 2020).

P. multocida is gram negative, facultative anaerobic and non-motile bacteria associated with animal diseases. *P. multocida* is the causative agent of Pasteurellosis which is considered as a contagious bacterial disease of rabbits, causing outbreaks and thus leading to economic losses in rabbit production investment sector (El-Jakee et al. 2020; Ismail et al. 2018).

Rabbit haemorrhagic disease virus and Pasteurellosis are fatal coinfections in rabbits and destructive for rabbits production exploited for human food and biomedical research. Both diseases induce a severe and frequently fatal infection. Many veterinarians in the profession are perplexed in differentiation between both diseases. Both have a short incubation period of 1-3 days, and animals might die. Both RHDV and *P. multocida* have a high mortality rate. Pasteurella, particularly *P. multocida* type A was often isolated from RHDV cases (Peshev and Christova 2003).

RHDV strains can be classified into three types based on phylogenetic analysis: classical RHDV with genogroups G1-G5, RHDV1, G6 variant strain (RHDV1) and the novel type RHDV2 (Qi et al. 2019). The variant strain RHDV1 was discovered in Egypt during 2006 (Salman 2007). Another variant known as RHDV2 was

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discovered in specific Egyptian governorates in 2018 and 2019, which was linked to much higher mortality rates, especially in suckling rabbits (Kwit and Rzeżutka 2019; Abido et al. 2020; Abodalal and Tahoon 2020 Erfan and Shalaby 2020; Hemida et al. 2020; Soliman et al. 2021). In 2019, in various parts of Upper Egypt, several RHDV1 strains were discovered and confirmed, posing a danger to the rabbit industry (Abodalal et al. 2021). It was recorded that the classical and variant RHDV1 vaccines were cross protective to each other (Abd El-Moaty et al. 2020), but there was no cross-protective effect between RHDV1and RHDV2 (OIE 2019).

Both RHDV and Pasteurella are the two major problems in industrial rabbitries and both of them are incriminated in a lethal hemorrhagic infection (simultaneous infection) in rabbitries (Ibrahim et al., 2021; O'Toole et al. 2022). For both RHDV and Pasteurella, the vaccination is made in order to increase the specific humoral immune response of the animal and protects against the two diseases. Rabbit breeders should vaccinate their rabbits at intervals against pasteurellosis and RHDV. A combined inactivated vaccine for the control of both diseases was used to decrease the stress factor of many vaccines injection (El-Maghraby et al. 2019). It should be taken into consideration that a combined inactivated vaccine against RHDV1 and *P. multocida* were prepared before, but the unique in the current study was that a single combined vaccine protects against both strains of RHDV (variant RHDV1 and RHDV2) and *P. multocida*. The prepared combined vaccine controls RHD viruses and Pasteurella in one shot consequently saving time, efforts of labor and avoiding stress of rabbits during vaccination (Peshev and Christova 2003). According to the findings, the bivalent vaccine candidate (RHDV1 and Pasteurella) not only reduced stress in rabbits by reducing the required manipulation by50%, but it also induced better protection and higher antibody response for both antigens. (El-Jakee et al. 2020). Primary goal of this study was to prepare an efficient combined vaccine which can be used as a single vaccination injection for protection of rabbits against RHDV and Pasteurella. This combined vaccine supply protection against these diseases, improves the immune response of the vaccinated rabbits, reducing the cost of vaccination, and decreasing different vaccination stress.

MATERIALS AND METHODS

Ethical Approval

The Veterinary Serum and Vaccine Research Institute (VSVRI) Abbasia, Agriculture Research Center, Ministry of Agriculture, Cairo, Egypt, approved this study. All procedures and rabbit care steps were carried out in accordance with the institutional guidelines for the use of animals in research.

Strains for the Vaccines Preparation

Rabbit Hemorrhagic Disease Viruses (RHDV)

Egyptian strains of RHDV1 and RHDV2 were provid ed by VSVRI, Abbasia, Cairo, Egypt. They were used for vaccine preparation, evaluation, and challenge and during HI test.

RHDV1

Local Egyptian strain of RHDV Giza/2006 has a titer of $10^{6.5}LD_{50}$/ml and a hemagglutination (HA) titer of 2^{14} HA unit**.**

RHDV2

Local Egyptian RHDV2 strain Mahala2019/VSVRI with Accession Number MK736667, titer of $10^{6.7}$ LD_{50}/ml and HA titer of 2^{12} HA unit.

P. Multocida

Local field isolates strains (serotypes A:1, A:3, A:12, D:2) were generously donated by the Aerobic Bacterial Vaccines Research Department, VSVRI, Abbasia, Cairo, Egypt.

Adjuvants

Montanide ISA 70 VG is a mineral oil-based adjuvant developed by SEPPIC Company, France for the production of water-in-oil (W/O) emulsion. It was used in accordance with the manufacturer's instructions.

Preparation of Inactivated Bivalent RHDV Montanide Vaccine

All procedures were carried out in accordance with the OIE (2018). Briefly, initially the RHDV1 and RHDV2 viruses were propagated in seronegative susceptible rabbits, and then the two viruses supernatants were individually inactivated for 48 hours at 37°C with formalin at a final concentration of 0.4%. The virus' inactivation was assessed by administering an inactivated suspension to five rabbits, with two rabbits as the control group. If the infected rabbits showed no clinical signs of illness or death, after that the vaccine adjuvant was to be used to emulsify the inactivated solution. Following that, Montanide ISA 70 VG adjuvant was added in accordance with the manufacturer's instructions (in a ratio of 70 adjuvant: 30 antigen).

Preparation of Inactivated Polyvalent *P. Multocida* Vaccine

According to Ismail et al. (2018), each serotype of *P. multocida* was grown individually in Tryptone Soya broth for 24 hours at 37°C aerobically. Each strain's concentration in the culture was adjusted to 4 x 109 CFU/mL. The culture was incubated for 24 hours at 37°C after being inactivated with 0.5% formalin. The cultures of each strain were combined in equal proportions. This inactivated culture was split into two parts. The initial one used for production of *P. multocida* Montanide ISA-70-VG to manufacturer instructions. The second part was used in preparation of the combined vaccine.

Preparation of Combined Inactivated RHDV1, RHDV2 and *P. Multocida* Vaccine

Using a magnetic stirrer, equal parts (V/V) of inactivated RHDV1, RHDV2, and *P. multocida* (serotypes A:1, A:3, A:12, D:2) were mixed. The fore mentioned suspension was adjusted to contain 4 x 10^9 CFU/mL of *P. multocida* according to Ismail et al. (2018) and with a titer of $10^{4.65}$ LD_{50}/ml and 2^{14} HAU/ml for RHDVa and RHDV2 at the final concentration of the end product.

Sterility Test

It was done in accordance with the British Veterinary Codex (2007). The prepared vaccines were examined for sterility (freedom from any bacterial or fungal contaminants) by cultivating on thioglycolate broth, MacConkey, and nutrient agar for 72 hours and about 1mL of the prepared vaccines was inoculated on Sabaroud dextrose agar for 15 days.

Safety Test

Safety test for prepared vaccines were carried out by S/C inoculation of 5 sero-negative rabbits with double doses of the recommended vaccinal dose for each prepared vaccine. The inoculated rabbits were observed for 2 weeks after inoculation (OIE 2018).

Experimental Design

A total of 230 two-month-old, New Zealand rabbits and weighting about 1.5kg were obtained from a private rabbitry that had no history of RHDV or *Pasteurella* outbreaks, nor was there any vaccination against them. They were kept in disinfected metal cages in a room that had good ventilation, where they were fed commercial pellets and given free access to clean water in VSVRI, Abbasia, Cairo. Firstly, it was confirmed that these rabbits were free from *P. multocida* and RHDV. They were carried out for vaccine preparation and evaluation.

The rabbits were divided into four groups: Group 1: Fifty rabbits were injected S/C with a dose of 1mL of RHDV inactivated bivalent oil adjuvanted vaccine per rabbit. Group 2: Fifty rabbits were injected S/C with inactivated Montanide *P. multocida* vaccine 1mL per rabbit. Group 3: Fifty rabbits were injected S/C with combined RHDV1 and RHDV2 and polyvalent Pasteurella vaccine 1mL per rabbit. Group 4: Twenty-five rabbits were injected S/C with 0.5mL of normal physiological saline per rabbit and maintained as non-vaccinated challenged (control +ve) group. Group 5: Twenty-five rabbits were injected S/C with 0.5mL of normal physiological saline per rabbit and maintained as non-vaccinated, unaffected (control -ve) group.

Collection of blood samples was done via ear vein weekly till 4th week post vaccination (WPV) then biweekly till 12th WPV then monthly till 6 months, for serum preparation to evaluate humoral immune response. At 3rd WPV, 20 rabbits from groups 1, 3 and 10 rabbits from group 4 were challenged with virulent RHDV1 and RHDV2 and 20 rabbits from groups 2, 3 and10 rabbits from group 4 were challenged with *P. multocida* types A and D.

Positive and Negative Control Serum of RHDV

Rabbit hemorrhagic disease viral antibody (RHDV-Ab) which used in HI test was supplied VSVRI, Abbasia, Egypt.

Evaluation of the Potency of the Vaccines For RHDV
Hemagglutination Inhibition (HI) Test

The serum sample was examined twice for RHDV1 RHDV1 and RHDV2 antigens. Serum samples were serially diluted twice in 50µL PBS before being incubated at 37°C for 30min. And the same amount of viral antigen with eight hemagglutinating units was added. Then 0.75% human type "O" RBCs were added (50µL) and incubated for 1 hour at 4°C. Mean HI $\log_2$/µL titers were used to determine the serum dilution that inhibited hemagglutination (OIE 2018).

Challenge Test

At the 3rd WPV, 20 rabbits were randomly selected from vaccinated groups 1, 3, and 10 rabbits from group 4. Selected rabbits were separated from each group into two subgroups of 10 rabbits each in order to complete the challenge test. Two RHDV viruses were given to each immunized group and each group was injected with a 1ml suspension of 10^3 LD_{50} virulent RHDV1 and 1ml suspension of 10^3 LD_{50} virulent RHDV2. For two weeks after the challenge, the rabbits were monitored daily. Post-mortem lesions and deaths were documented (OIE 2018; OIE 2021).

For *P. Multocida* Evaluation Indirect Hemagglutination Test (IHA): According to (OIE 2013).

Challenge Test

After the third week, 20 rabbits from each vaccinated group (2 and 3) and 10 rabbits from group 4 were selected at random relocated to experimental isolators and challenged with 0.2mL of 10^8CFU of *P. multocida* cell suspension serotypes A and D. Rabbits were observed for 14 days after the challenge (Ismail et al. 2018).

RESULTS AND DISCUSSION

The rabbit industry is one of the small livestock industries that can share in the solving of animal protein deficiency problem in the developing countries (Cullere and Dalle 2018; Trocino et al. 2019; Sikiru et al. 2020; Al-Ebshahy et al. 2022). RHDV is a contagious and fatal viral illness that affects rabbits and causes outbreaks in the rabbit population around the world. (El-Samadony et al. 2021). Pasteurella multocida is the most common bacterial pathogen isolated from rabbits. It is the cause of a highly contagious disease of rabbits, snuffles, which primarily affects the upper respiratory tract with potentially fatal consequences including otitis media; enzootic pneumonia, conjunctivitis, pyometra, orchitis, abscesses, and septicemia (El-Jakee et al. 2020).

So, it is very important to protect rabbit industry from these diseases threatening it, RHDV (1, 2) and rabbit Pasteurellosis. This can be achieved by strict vaccination program against these diseases. So, it is very important to protect rabbit industry from the two well-known diseases threatening i.e., RVHD and rabbit Pasteurellosis (Rocchi et al. 2019; Agüero et al. 2019; Zhu et al. 2020; Yang et al. 2022). This can be achieved by strict vaccination program against these two diseases. Nowadays, combined veterinary vaccines against more than one disease either such disease is bacterial, viral or bacterial and viral became available to avoid the stress of manipulation during vaccination (El-Maghraby et al. 2019). Our study aimed to achieve these goals through preparing monovalent and combined vaccines of excellent potency and safety by using Montanide oil which enhances the immune response to control such diseases.

The vaccines were found to be sterile (no growth of micro-organisms on nutrient agar, blood agar and Sabouraud agar). During the three weeks of observation, the ten S/C inoculated rabbits did not develop any abnormal local or systemic reactions. These findings agree with those recommended by OIE (2018).

During this study, three types of vaccines were administered to three groups of rabbits. The first group received a bivalent Montanide adjuvanted inactivated RHDV vaccine. Group 2 received Montanide adjuvanted inactivated *P. multocida* vaccine, while Group 3 received a combination of Montanide adjuvanted inactivated RHDV1and RHDV2 and *P. multocida* vaccine.

As shown in Table 1, none of the vaccinated and control rabbits had RHDV specific HI antibodies before vaccination then high titers of RHDV specific HI antibody were detected in sera of vaccinated rabbits beginning with the 1st week post vaccination (WPV) in the different groups. It was noticed that HI RHDV-antibodies titer induced by oil emulsified vaccine ranging from 2^6 to $2^{6.5}$ for RHDV1 and RHDV2, respectively, at 1 WPV in group 1. These results agree with Abodalal et al. (2022**)** who reported a value of 2^6 for the RHDV1and higher ($2^{5.75}$) for the RHDV2 at 1 WPV in rabbits vaccinated with bivalent inactivated oil vaccine. Salman (2007) reported that rabbits vaccinated by inactivated RHDV1 vaccine adjuvanted with Montanide ISA 71 gave $2^{\mathbf{4.25}}$ antibody titer i.e., lower than the antibody titers obtained from the prepared combined vaccine.

There was a gradual increase in antibodies against RHDV HI (RHDV1 and RHDV2) in the two vaccinated groups and the peak was attained at12th WPV then gradually decreases till 24 weeks but still high and protective, based on the previous results for Group 3 and Group 1, it was concluded that these two groups produced similar results.

P. multocida is a serious bacterial infection that causes significant economic losses in rabbits. Controlling that disease is still a topic of interest and draws the attention of many researchers. Vaccination is one of the most significant methods for preventing the disease. In the present study as shown in Table 3, none of the serum samples from all vaccinated and control rabbits showed presence of antibodies against *P. multocida* before vaccination. All vaccinated rabbits in groups 2 and 3 induced a systemic humoral antibody as measured by indirect hemagglutination test.

It was clear from the findings of the current study as seen from Table 3, the group 2 which was vaccinated with inactivated *P. multocida* Montanide 70 gave lower antibody titer against *P. multocida* type A and D (8 and 8.5 respectively) at 1st WPV than group 3 (vaccinated with combined Montanide 70 inactivated vaccine) which gave 9.2 and 9.5 titers of antibodies against *P. multocida* types A and D respectively. This result was supported by El-Maghraby et al. (2019) who found that early and high immune response occurred in rabbits vaccinated with combined RHDV1, Pasteurella and Clostridia vaccine contained Montanide oil. and this agree with Ismail et al. (2018) while El-Maghraby et al. (2019) reported that the mean IHA antibody titer of *P. multocida* in the sera of rabbits vaccinated with Montanide oil adjuvanted *P. multocida* and combined vaccines at the 2 weeks after vaccination increased gradually till reached the maximum level at 8th week post and decreased slightly from the 10th week till the end of the experiment.

In groups 2 and 3, the antibody titers increased from the first week after vaccination until they reached the maximum level (18.98,18) and (18.5,18.2) for the serotypes A and D of *P. multocida* at the 6th week then slightly reduced from the 8th week till the end of experiment and this coincided with the findings of Ismail et al. (2018) while El-Maghraby et al. (2019) found a mean IHA antibody titer of *P. multocida* in vaccinated rabbit sera at the 2 weeks after vaccination gradually increased until the eighth week then decreased slightly from the 10th week till the end of the experiment.

The present outcomes were supported with the findings of Abd El-Aziz et al. (2015), who reported that, inactivated *P. multocida* vaccine adjuvanted with Montanide ISA-70-VG vaccine elicited an early and strong immune response. In addition, Ahmed et al. (2010) concluded that the inactivated *P. multocida* vaccine adjuvanted with montanide was effective and had high antibody titers measured by IHA.

Also, Ibrahim et al. (2021) concluded that the inactivated *P. multocida* Montanide adjuvanted vaccine gave long duration antibody titer when assessed by IHA test. Youssef and Tawfik (2011), when used the IHA test, found that inactivated rabbit Pasteurella vaccine

Table 1: Geometric means of rabbit hemorrhagic disease virus-specific antibody titers (log2) in the sera of vaccinated and unvaccinated rabbits

Post vaccination period	Geometric means of RHDV HI antibody titers (log2)					
	Group1		Group2		Group3	
	RHDVa	RHDV2	RHDVa	RHDV2	RHDVa	RHDV2
Day 0	0	1	1	0	0	0
1WPV	6	6.5	5	5.5	2	0
2 WPV	6.5	6	6	5.75	1	1
3 WPV	6.5	6.5	7	6	1	1
4 WPV	7	7.2	7.5	7.75	2	0
6 WPV	7.2	7.2	8	8.2	0	0
8 WPV	8	8.2	9	9.5	0	0
10 WPV	10	10.25	11	11.25	0	0
12 WPV	10.5	11	11.75	11.5	1	1
16 WPV	10.5	10	11.5	11	2	1
20 WPV	9.5	9	10	10	1	0
24 WPV	9	8.5	10.5	10	0	0

Group 1: vaccinated with montanide oil bivalent RHDV vaccine. Group 3: injected with combined montanide oil vaccine. Group 4: kept as non-vaccinated group.

Table 2: Protective efficacy in rabbits vaccinated with bivalent RHDV and combined vaccines adjuvanted with montanide oil against virulent RHDV strains

Groups	Challenge Ag	Total number	Number of survived /total number of rabbits	Protection %
1	RHDV1	10	9/10	90
	RHDV2	10	8/10	80
3	RHDV1	10	9/10	90
	RHDV2	10	10/10	100
4	RHDV1	5	0/10	0
	RHDV2	5	0/10	0

Group 1: vaccinated with montanide oil bivalent RHDV vaccine.
Group 3: injected with combined montanide oil vaccine.
Group 4: kept as non-vaccinated challenged (control +ve) group.

Table 3: Results of Anti-*Pasteurella multocida* antibodies in sera of rabbits vaccinated with monovalent and combined inactivated with montanide oil ISA70 by IHA test

Post vaccination period	Geometric means of *P. multocida* IHA antibody titers					
	Group 2		Group 3		Group 4	
	A	D	A	D	A	D
Day 0	2	2	2	2	2	2
1WPV	8	8.5	9.2	9.5	2	2.5
2 WPV	9.2	9.5	9.5	10	2	2.3
3 WPV	9.5	10	10	10.97	2	2.16
4 WPV	10	10.5	10	11	2	2.12
6 WPV	18.98	18	18.5	18.2	2	2
8 WPV	17.33	17	17	17.2	2	2
10 WPV	15.33	15.5	15.5	15.6	2	2
12 WPV	14.98	15	14.98	15.3	2	2
16 WPV	14.26	14.5	13.5	14.6	2	2
20 WPV	13.66	14	11.97	13.5	2	2
24 WPV	10.97	11	10.5	11	2	2

Group 2: vaccinated with montanide oil *P. multocida* vaccine.
Group 3: injected with combined montanide oil vaccine.
Group 4: kept as non-vaccinated group.

Table 4: Protective efficacy in rabbits vaccinated with different prepared vaccines against *P. multocida*

Groups	Challenge Ag	Total number	No. survived /total number of rabbits	Protection %
2	A	10	8/10	80
	D	10	8/10	80
3	A	10	9/10	90
	D	10	9/10	90
4	A	5	0/5	0
	D	5	0/5	0

Group 2: vaccinated with montanide oil *P. multocida* vaccine. Group 3: injected with combined montanide oil vaccine. Group 4: kept as non-vaccinated challenged (control +ve) group.

adjuvanted with Montanide ISA-50 produced protective antibody titers against *P. multocida* and gave high and long-lasting antibody levels.

The results of challenge test have been illustrated in Table 4, the protection percentage (P%) against the challenge with *P. multocida* type "A" for rabbit groups was 80% for the group vaccinated with *P. multocida* vaccine (group 2) and 90% for the combined one (group 3) but 0% for control group (group 4).

While protection % against *P. multocida* type "D" challenge was 80% in rabbit groups vaccinated with *P. multocida* vaccine and 90% for the combined one in comparison with 0% for control group. These findings were consistent with previous studies (Fatma Fathy 2018; El-Maghraby et al. 2019; Ibrahim et al. 2021) who found that *P. multocida* adjuvanted vaccines were more effective against challenge with virulent strains of *P. multocida* types A and D.

We concluded that the best vaccine in induction of high and sustained immune response against both of RHDV1and RHDV2 and *P. multocida* is the combined oil inactivated RHDV1and RHDV2 and *P. multocida*.

Conclusions

The combination vaccine against RHDV1 and RHDV 2 and Pasteurellosis, appears to be safe, with a combination of speed and longevity in the immunological response. Immune protection against RHDV1 and RHDV 2 and Pasteurellosis can be achieved with a single manipulation, reducing animal stress and effort.

Conflict of Interest

According to the authors, the current study was conducted without any conflicts of interest.

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Authors contribution

Samah El Sayed Abodalal came up with the idea for the article. The experiment was done, and the manuscript was written, designed, and carried out by all of the authors.

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