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# Efficacy of an inactivated bivalent Rabbit Hemorrhagic Disease Virus 2 – Pasteurellosis vaccine in Rabbits by using a new Adjuvant

**Samah El Sayed Ali Abodalal[1]*, Amina A. Radwan[2] and Mahmoud T. A. Ismail[3]**

*1 Poultry Viral Vaccine Department, Veterinary Serum and Vaccine Research Institute (VSVRI), Abbasia, Agriculture Research Center (ARC), Cairo, Egypt*

*2 Central Laboratory for Evaluation of Veterinary Biologics (CLEVB), Abbasia, Egypt.*

*3 Aerobic Bacteria Research Department, Veterinary Serum and Vaccine Research Institute (VSVRI), Abbasia, Agricultural Research Center (ARC), Cairo, Egypt*

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**ABSTRACT**

The purpose of this study is to prepare an efficient vaccine combating Rabbit Hemorrhagic Disease Virus (2) (RHDV2) and Pasteurellosis (serotypes A:1, A:3, A:12, D:2) using Coral vac RZ 528 adjuvant. Four groups of rabbits were used to evaluate the efficiency of this vaccine. Groups were injected as follows: 1 with coral oil-based RHDV2 vaccine, 2 with coral oil-based *P. multocida* vaccine, 3 with the adjuvanted coral oil-based combined vaccine, and 4 was kept as a control group not injected. The efficiency of the prepared vaccines was assessed through Hemagglutination Inhibition (HI) and challenge tests for RHDV2, as well as indirect Hemagglutination Inhibition (indirect HI) and challenge tests for *P. multocida*. The HI test results showed that the 12th week after vaccination was the peak in antibody titers (11.5 for the RHDV vaccine and 11.8 for the combined one). The challenge test showed that both vaccines were 100% protective. For *P. multocida* and the combined vaccines, the evaluation was done by the Indirect Hemagglutination (IHA) test and challenge test. By IHA test, the 6$^{th}$ week was the highest in the antibody titers (for *P. multocida* vaccine 1638.4 for A, 1433.6 for D serotypes, and the combined one 2048 for A, 1843.2 for D). The challenge test result was a 90% protection rate for both vaccines. In conclusion, the combined prepared vaccine is efficient and protective against both diseases, with the advantages of decreasing the stressors on the animals and the workers and lowering the cost.

## 1. INTRODUCTION

Rabbit hemorrhagic disease and Pasteurellosis are deadly co-infections that harm rabbit production, which is used for human consumption and scientific study. Both illnesses cause a serious infection that is often fatal. Animals may perish during the brief incubation period of one to three days for both. The mortality rate for *Pasteurella multocida* (*P. multocida*) and RHDV is significant. Pasteurella was frequently recovered from Rabbit hemorrhagic disease virus (RHDV) cases, especially *P. multocida* type A (Peshev and Christova, 2003).

Rabbit hemorrhagic disease virus (RHDV) harms the Egyptian economy and has resulted in deterioration in the rabbit production, resulting from high morbidity and mortality. Ninety % of both domestic and wild rabbits die from RHDV, an acute and rapidly spreading virus that affects rabbits all over the world. RHDV is a member of the Caliciviridae family, genus Lagovirus. The virus is an icosahedral, single-stranded, positive-sense RNA virus that is not enclosed. Phylogenetic analysis allows for the classification of RHDV strains into three kinds (Abodalal and Ismail, 2023). In 2018 and 2019, a mutant strain of RHDV2 was identified in several Egyptian governorates and was associated with significantly higher fatality rates, particularly in suckling rabbits (Abodalal and Ismail, 2023).

Pasteurella multocida (*P. multocida*) is the causative agent of rabbit pasteurellosis. It is a member of rabbit's critical bacterial diseases that cause investment losses in the production scale all over the world (Abd El-Samie, 2014). Rhinitis, pneumonia, visceral and subcutaneous abscessation, metritis, orchitis, septicemia, and otitis media can all result from subclinical Pasteurella infections. The infection usually initiates in the upper respiratory tract. Transmission is facilitated by direct exposure between the susceptible and the carriers. The transmission by the airborne way after 21 days of exposure does not occur. High air ammonia concentrations and transportation frequently encourage latent *P. multocida* to flourish and spread illness (Suckow et al., 2008). To protect rabbits from pasteurellosis, several vaccines have been investigated, including ones made of complete, inactivated bacteria. As the two main issues in industrial rabbitries are RHDV and Pasteurella, both of which are implicated in a fatal hemorrhagic illness (simultaneous infection) in rabbits, both RHDV and Pasteurella are protected against by the vaccination, which is designed to boost the animal's particular humoral immune response. Breeders of rabbits should periodically vaccinate their animals against RHDV and pasteurellosis (El-Maghraby et al., 2019). The stress factor of frequent vaccine injections was reduced by using a combination inactivated vaccine for the management of both illnesses (El-Maghraby et al., 2019).

Therefore, the purpose of this research is to create a successful and effective inactivated vaccination against RHDV2 and *P. multocida* in rabbits by using Coralvac RZ 528 adjuvant.

* Correspondence to: drsamahsaidvet@gmail.com

## 2. MATERIAL AND METHODS

*2.1 Ethical approval*

The Veterinary Serum and Vaccine Research Institute (VSVRI), Abbasia, Agriculture Research Center, Ministry of Agriculture, Cairo, Egypt, approved this study. The institutional rules for the use of animals in research were followed at every stage of the rabbit care process.

*2.2 Strains for the vaccine preparation*

*2.2.1 Rabbit hemorrhagic disease virus 2*

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, was provided by VSVRI, Abbasia, Cairo, Egypt. It was used for vaccine preparation and evaluation using the HI test and the challenge test.

*2.2.2 Pasteurella multocida*

Local field isolates strains (serotypes A:1, A:3, A:12, D:2; containing 4 x $10^9$ CFU/mL) were generously donated by the Aerobic Bacterial Vaccines Research Department, VSVRI, Abbasia, Cairo, Egypt.

*2.3 Adjuvant*

The Turkiya Company developed Coralvac RZ 528, a mineral oil-based adjuvant, to create a water-in-oil (W/O) emulsion. The usage followed the guidelines provided by the manufacturer.

*2.4 Preparation of Inactivated RHDV2 Coralvac RZ 528 vaccine*

All procedures are in accordance with the OIE (2018). Briefly, the RHDV2 virus was initially propagated in seronegative susceptible rabbits by the intramuscular route, then the virus was harvested from the liver after 3 days post-injection, and then the virus supernatant at 37°C was inactivated for 48 hours with a concentration of 0.4% formalin. The inactivation was evaluated by administering the inactivated suspension to five rabbits by the intramuscular route, with two rabbits as the control group. If the infected rabbits do not show clinical signs of illness or death, the vaccine adjuvant is to be used to emulsify the inactivated solution. Following that, Coral VAC 528 RZ adjuvant was added in accordance with the manufacturer's instructions (70 adjuvant: 30 antigen).

*2.5 Preparation of inactivated polyvalent P. multocida Vaccine According to Ismail et al. (2018)*

Briefly, each species of *P. multocida* was cultivated separately in tryptone soya broth at 37°C aerobically for 24 hours, then inactivation with 0.5% formaldehyde. The concentration of the bacterial culture was adjusted to be 4 × $10^{\wedge 9}$ CFU/mL for both vaccines in the final vaccine dose. This inactivated culture was divided into two parts; The first part was used to produce *P. multocida* Coral VAC 528 RZ adjuvant was added following the manufacturer's instructions (70 adjuvant: 30 antigen). The second part was used to prepare the combined one.

*2.6 Preparation of Combined Inactivated RHDV2 and P. multocida vaccine*

Using a magnetic stirrer, the equivalence(V/V) of the inactivated RHDV2, P. multocida culture, and Coral VAC 528 RZ adjuvant was added following the manufacturer's instructions (70 adjuvant: 30 antigen), was mixed. The final vaccinal dose was adjusted to contain 4 × $10^9$ CFU/mL for *P. multocida*, and RHDV2 titer was $10^{4.65}$ $LD_{50}$/mL and $2^{14}$ HAU/mL

*2.7 Quality control tests*

*2.7.1 Sterility test*

Following the British Veterinary Codex (2007), from the prepared vaccines, 1 ml was applied for 72 hr. on nutrient agar, MacConkey agar, and thioglycolate broth, then for 15 days on Sabouraud dextrose to check the sterility (confirmation of no bacterial or fungal contamination). Additionally, Mycoplasma agar was cultivated to identify Mycoplasma contamination, and it was cultured for 14 days at 37 °C with 5% $CO_2$.

*2.7.2 Safety test*

Five seronegative rabbits were injected S/C with double the recommended dosage for each manufactured vaccine. For 2 weeks, the injected rabbits were observed, in accordance with the (OIE 2018).

*2.8 Experimental design*

A total of 105 two-month-old rabbits from New Zealand, about 1.5kg in weight, were obtained from private rabbit farms with no history of RHDV or Pasteurella outbreaks or vaccinations. They were kept in a well-ventilated, disinfected metal cage, provided with commercial pellets, and accessed free clean water in the VSVRI, Abbaasia, Cairo. First, it was confirmed that these rabbits were free from *P. multocida* and RHDV. These rabbits were used for preparing and evaluating the vaccines. The rabbits were divided into four groups:

Group (1): 30 rabbits were vaccinated S/C with 1 ml of RHDV2 inactivated Coral VAC 528 RZ adjuvanted vaccine per rabbit.

Group (2): 30 rabbits were vaccinated S/C with inactivated Coral VAC 528 RZ adjuvant *P. multocida* vaccine, 1 mL per rabbit.

Group (3): 30 rabbits were vaccinated S/C with Coral VAC 528 RZ adjuvant combined with RHDV2 and polyvalent Pasteurella vaccine, 1ml per rabbit.

Group (4): 15 rabbits were kept as non-vaccinated, challenged control group.

Collection of blood samples was done via ear vein weekly till the 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 week post vaccination (WPV), 10 rabbits from groups 1,3 (vaccinated groups) and 5 rabbits from group 4 (control group) were challenged with virulent RHDV2 and 10 rabbits from groups 2, 3 (vaccinated groups) and 5 rabbits from group 4 (control group) were challenged with *P. multocida* types A and D.

*2.9 Evaluation of the potency of the vaccines*

*2.9.1 For Rabbit hemorrhagic disease virus*

*2.9.1.1 Hemagglutination inhibition (HI) test*

Two analyses were performed on the serum sample to check for RHDV2 antigens. They were incubated for 30 minutes after serial diluting serum samples twice in 50µL PBS at 37°C. Eight hemagglutinating units with the same quantity of viral antigen were also added, then the addition of 0.75% human type "O" RBCs (50µL) and incubation for 1 hour at 4°C were done. Mean HI $\log_2$/µL titers were used to determine the serum dilution that inhibited hemagglutination (OIE 2018, OIE 2021).

*2.9.1.1.1 Positive and negative control serum of RHDV*

RHDV antibody (RHDV-Ab) ($2^{10}$ HI unit), which was utilized in the HI test, was given by VSVRI, Abbasia, Egypt.

*2.9.1.2 Challenge test*

At the 3rd WPV, 10 rabbits were randomly selected from vaccinated groups 1, 3 (10 rabbits per group), and 5 rabbits from group 4. Selected rabbits were injected intramuscularly with a 1 ml 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).

*2.9.2 For P. multocida evaluation*

*2.9.2.1 Indirect Hemagglutination Test (IHA)*

IHA test was performed according to (OIE 2013), in short, 50 µl of sensitized RBCs were added to each well after two-fold serial dilutions of the collected sample sera, commencing with 1/2, were made in stabilizer buffer pH 7.2 to reach a final amount of 50 µl/well (microtiter plate). Sensitized erythrocytes plus diluent and unsensitized erythrocytes plus test serum served as controls. The first reading was obtained after shaking the plates and letting them sit at room temperature for about two hours. After that, the plates were kept in the fridge until the second reading was made the following morning.

*2.9.2.2 Challenge test*

After the third week, 10 rabbits from 2 and 3 groups (vaccinated groups) and 5 rabbits from group 4 (control group) were selected at random manner, relocated to experimental isolators and challenged with 0.2mL of $10^8$CFU of *P. multocida* cell suspension serotypes A and D. Rabbits were observed for 14 days after the challenge (Ismail et al., 2018).

## 3. RESULTS

*3.1. Quality control testing of the prepared vaccines*

The sterility tests showed that all the prepared vaccines were free from bacterial, fungal, and mycoplasma contamination. The prepared vaccines were proven to be safe; there were no local or systemic post-injection reactions for 15 days of clinical observation.

*3.2 Humoral immune response against RHDV2 vaccines using the HI test*

According to Table 1, the antibody titers began to increase from the first week after vaccination till the 12th week post vaccination, achieving the highest antibody titers for both RHDV2 vaccines (11.5 for RHDV2 vaccine, 11.8 for the combined one).

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

| Post vaccination Period | Means of RHDV HI antibody titers (log2) | | |
|---|---|---|---|
| | Group1 | Group3 | Group 4 |
| Day 0 | 0 | 0 | 0 |
| 1WPV | 6 | 6.2 | 0 |
| 2 WPV | 7 | 6.5 | 1 |
| 3 WPV | 7.2 | 7 | 1 |
| 4 WPV | 7.5 | 8.5 | 0 |
| 6 WPV | 8 | 9 | 0 |
| 8 WPV | 8.5 | 10 | 0 |
| 10 WPV | 10 | 11.2 | 0 |
| 12 WPV | 11.5 | 11.8 | 1 |
| 16 WPV | 11 | 11.5 | 1 |
| 20 WPV | 11.2 | 10.5 | 0 |
| 24 WPV | 10 | 10.2 | 0 |

WPV: weeks post vaccination, Group 1: vaccinated with coral oil RHDV2 vaccine, Group 3: injected with the combined vaccine, Group 4: control group.

*3.3 Efficacy of the prepared vaccines using the challenge test for RHDV2*

According to Table 2, which revealed the Challenge test results for estimating in rabbits immunized against a virulent strain of RHDV2, 100% protectivity was demonstrated by the RHDV2 and the combined vaccine adjuvanted with Coral oil.

Table 2 Protectivity in the vaccinated groups with RHDV2 and combined vaccines adjuvanted with coral oil against the virulent RHDV2 strain

| Groups | Total number | Number of survived /total number of rabbits | Protection % |
|---|---|---|---|
| 1 | 10 | 10/10 | 100 |
| 3 | 10 | 10/10 | 100 |
| 4 | 5 | 0/5 | 0 |

Group 1: vaccinated with Coral oil bivalent RHDV vaccine, Group 3: injected with the combined vaccine, Group 4: control group.

*3.4 Humoral immune response against P. multocida vaccines using the indirect HI test*

According to Table 3 by IHA test, the mean of the antibody titers began to increase from the 1st week after vaccination till the 6th week for *P. multocida* vaccine (A titer was 1638.4 and D titer was 1433.6) and for the combined one (A titer was 2048 and D titer was 1843.2). After that, the antibody titers began to decrease.

*3.5 Efficacy of the prepared vaccines using the challenge test for P. multocida*

According to table (4) by challenge test, the protective percentage for both *P. multocida* vaccines was 90% for both serotypes (A and D). But for the control group, the protective percentage was 0%.

Table 3 Antibody titers against *P. multocida* in vaccinated rabbits' sera with *P. multocida* and the combined vaccines by IHA test

| Post-vaccination period | Means of *P. multocida* IHA antibody titers | | | | | |
|---|---|---|---|---|---|---|
| | Group 2 | | Group 3 | | Group 4 | |
| | A | D | A | D | A | D |
| Day 0 | 2.4 | 2 | 2 | 2 | 2.4 | 2 |
| 1WPV | 384 | 358.4 | 460.8 | 409.6 | 2.4 | 2.4 |
| 2 WPV | 614.4 | 512 | 819.2 | 716.8 | 2 | 2 |
| 3 WPV | 921.6 | 819.2 | 1228.8 | 1024 | 2 | 2 |
| 4 WPV | 1024 | 921.6 | 1433.6 | 1228.8 | 2.4 | 2.4 |
| 6 WPV | 1638.4 | 1433.6 | 2048 | 1843.2 | 2 | 2 |
| 8 WPV | 1024 | 921.6 | 1433.6 | 1228.8 | 2 | 2 |
| 10 WPV | 819.2 | 716.8 | 1126.4 | 921.6 | 2.4 | 2 |
| 12 WPV | 512 | 460.8 | 716.8 | 614.4 | 2 | 2 |
| 16 WPV | 460.8 | 409.6 | 614.4 | 512 | 2 | 2 |
| 20 WPV | 307.2 | 256 | 409.6 | 358.4 | 2 | 2 |
| 24 WPV | 204.8 | 179.2 | 281.6 | 230.4 | 2 | 2.4 |

Group 2: vaccinated with Coral oil *P. multocida* vaccine, Group 3: vaccinated with the combined vaccine, Group 4: control group.

Table (4) showing the Protective efficacy in rabbits vaccinated with different prepared vaccines against *P. multocida*

| Rabbits Groups | Challenge Ag | Total number | Number of survived /total number of rabbits | Protection % |
|---|---|---|---|---|
| 2 | A | 10 | 9\10 | 90 |
| | D | 10 | 9\10 | 90 |
| 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 Coral oil *P. multocida* vaccine.
Group 3: vaccinated with the combined vaccine.
Group 4: control group.

## 4. DISCUSSION

One of the small livestock sectors that can help combat the issue of animal protein shortages in developing nations is the rabbit industry. Therefore, protecting the rabbit investment from illnesses like rabbit pasteurellosis and RHDV is crucial. Strict immunization against these infections can help achieve this. That is why protecting the rabbit industry from the two known illnesses that pose a threat, rabbit RHD and pasteurellosis, is crucial (Abodalal and Ismail, 2023). RHDV is a destructive and infectious virus that infects rabbits and leads to epidemics in the

global rabbit population (El-Samadony et al., 2021). The most frequent bacterial pathogen found in rabbits is *Pasteurella multocida*. Mostly affecting the upper respiratory tracts of rabbits, it is the cause of snuffles, a highly contagious disease that can lead to a number of disastrous disorders, such as septicemia, pyometra, otitis media, enzootic pneumonia, conjunctivitis, orchitis, abscesses, and pyometra (El-Jakee et al., 2020). Strict immunization campaigns against these two illnesses can help achieve this. These days, it is possible to prevent the stress of vaccination by using veterinary vaccinations that protect against multiple diseases, whether they are bacterial, viral, or both (El-Maghraby et al., 2019). By employing Coralvac RZ 528 mineral oil, which boosts the immune response to control such diseases, our study sought to accomplish these goals by creating monovalent and combination vaccinations with exceptional potency and safety.

In this study, for all the prepared vaccines during three weeks of observation, the inoculated rabbits did not exhibit any abnormal local or systemic reactions, and the vaccines were approved to be sterile. These results are consistent with those suggested by OIE (2018).

Hemagglutination inhibition (HI) test was used for evaluating the developed immunity against RHDV2 in the prepared vaccines. The present results are coordinated with those reported by Abodalal and Ismail (2023) who prepared a combined Vaccine against RHDV (1 and 2) and *P. multocida* adjuvanted with Montanide ISA 70 VG, as they cited that the 12$^{th}$ week was the highest in the antibody titers and the protectivity were 80% for RHDV2 vaccine and 100% for the combined one. According to Salman (2007), Adjuvanted with Montanide ISA 71, rabbits administered the inactivated RHDV1 vaccination generated antibody titers of 24.25, which was less than the antibody titers from the prepared combined vaccine. El-Maghraby et al. (2019) cited that the 10$^{th}$ week post boostering was the highest in the antibody titers (11.5 for RHDV2 vaccine, 12 for the combined one), and the combined inactivated vaccine they prepared, which included *P. multocida*, *C. perfringens* (A), and the RHDV, resulted in 100% protectivity.

For *P. multocida* vaccine evaluation, the IHA test and challenge test were used to evaluate both the *P. multocida* vaccine and the combined one. Similar to our results, El-Maghraby et al. (2019) developed an inactivated vaccine that contains the viruses that cause RHD, *C. perfringens* (A) and *P. multocida*. They found that after evaluating the Pasteurella vaccines adjuvanted with Montanide oil ISA 70 by IHA test, the 8$^{th}$ week post the booster dose was the highest week in the antibodies' titer for Pasteurella vaccine (2048 for both serotypes A and D) and for the combined one (2090, 2059 respectively for serotypes A and D) and the protective efficacy by challenge test was 90% for the Pasteurella vaccine and 100% for the combined vaccine. They concluded that the combined vaccines were safe and that the combination enhances the immune response and decreases the stress on animals and efforts. Abodalal and Ismail (2023) also prepared an inactivated combined vaccine of rabbit hemorrhagic disease virus types 1 and 2 and *P. multocida* adjuvanted with Montanide oil ISA 70. IHA test results showed that the 6$^{th}$ week post-vaccination was the highest in the titers of antibodies. The protection rate was 80% for the Pasteurella vaccine and 90% for the combined one. According to their results, they approved the combination against RHDV1 and RHDV2 and Pasteurellosis vaccine, which appears to be safe, with a rapid, long-lasting immunity in a single manipulation, saving the repetition of the vaccination procedures. On the same approach, Youssef and Tawfik (2011) cited that the inactivated adjuvanted *P. multocida* vaccine produced the highest protective antibody titers between the 6th and the 8th week when evaluated by ELISA assay and IHA test. They also evaluated the protection rate by the challenge test, which revealed the protection % was between 80:100%. Mohamed et al. (2024) prepared different inactivated rabbit Pasteurellosis vaccines with different adjuvants (Montanide ISA206 and Carbopol®) to compare them and vaccinated the rabbits with 2 doses, then challenged them. They used the IHA and challenge test for estimating the efficacy of the vaccines. The highest titers for these vaccines were at the 6$^{th}$ week after the challenge test (1024 for serotype A and 512 for serotype D). Then decreased to reach 128 at the 14$^{th}$ week after the challenge, for serotype A and also for serotype D was 32. The protection rate ranged from 93% to 100%. Alsakini et al. (2024) used the same adjuvant as that used in this study (Coralvac RZ 528) and explained that the Coral Biotechnology-developed adjuvants and the humoral and cellular immune responses enhanced by these novel adjuvants for vaccination would make adjuvant vaccines. Zyan et al. (2004) conducted a field trial to evaluate the efficacy of the Aluminum Hydroxide gel rabbit Pasteurella vaccine (ALV) in rabbits. They found that rabbits vaccinated with two doses of ALV and one dose of oil adjuvant bacterins had good protection levels (83.3%) and high levels of indirect hemagglutination antibody titer (IHA), while rabbits vaccinated with a single dose of ALV only showed 33.3% protection.

## 5. CONCLUSIONS

The combined inactivated vaccine against RHDV2 and Pasteurellosis, adjuvanted with Coral VAC 528 RZ adjuvant, appeared to be safe and induce rapid immunity. The protection against RHDV2 and *P. multocida* achieved with one injection decreases the cost, stressors, and efforts.

## CONFLICT OF INTEREST

There are no conflicts of interest in this paper, according to the authors.

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