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The use of different types of Montanide adjuvant in preparation of inactivated rabbit haemorrhagic disease virus (RHDV) vaccine

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Abstract

Background: Rabbit viral haemorrhagic disease (RVHD) is characterized by high mortality in adult rabbits causing severe economic losses. Vaccination against RHDV is the main preventive method.

Objective: The current study was conducted to investigate the immuno-enhancing effects of some Montanide incomplete seppic adjuvants (ISAs) incorporated within inactivated RHDV vaccine on vaccinated rabbits in comparison to the currently produced aluminum hydroxide (Al (OH)3) gel adjuvanted vaccine.

Methods: Four experimental batches of inactivated RHDV oil emulsified (OE) vaccines were prepared using 4 different types of Montanide ISAs (SEPPIC, France) (ISA 70 VG, ISA 71 VG as water-in-oil emulsion (W/O), ISA 206 VG as water-in-oil-in-water emulsion (W/O/W), and ISA 760 VG as water-in-polymer emulsion (W/P)) in addition to another batch adjuvanted with Al $(OH)_3$ gel. The efficacy of the prepared vaccines was studied in five groups of vaccinated rabbits. The efficacy was based on humoral immune response measured by HI test and ELISA in addition to the resistance to challenge with virulent RHDV.

Results: All of the prepared vaccines induced specific RHDV-antibodies, with variable titers, detected from the 1st week post vaccination (WPV) in vaccinated rabbits. The induced RHDV-antibody titers increased to reach their highest level in 3rd month post vaccination (MPV) for all groups except groups (2) (vaccinated with Montanide ISA 70 adjuvanted vaccine) and (5) (vaccinated with Montanide ISA 760 adjuvanted vaccine) in which the maximum level was attained at 6th WPV and 4th MPV respectively. The vaccinated rabbits resisted the challenge against virulent RHDV as early as at the 3rd WPV and at 6th MPV with 100% protection in contrary to unvaccinated group in which all rabbits get died (0% protection).

Conclusion: Montanide ISAs used in this study could be used to produce inactivated RHDV OE vaccine and found to be more preferable than Al $(OH)_3$ gel in enhancing the immune response of vaccinated rabbits achieving higher and longer term protective immune responses with preference of the following order, Montanide ISA 70, 206, 760 and finally 71.

Key words: Inactivated Oil Emulsion vaccine; Montanide ISAs; Rabbit Haemorrhagic Disease Virus; HI; ELISA

Introduction

Rabbit viral haemorrhagic disease (RVHD) is a highly contagious and acute fatal disease of the European rabbit (Oryctolagus cuniculus), caused by a calicivirus (genus Lagovirus) and characterized by high morbidity and high mortality rates (70–90%) **(OIE, 2014)**. So, RVHD is of a high economic importance in the rabbit industry. RVHD is a worldwide disease and it was first described in China in 1984 **(Liu, et al., 1984)**, while in Egypt, it was first recorded in 1991**(Ghanem and Ismail, 1992)**. RVHD controlling key in domestic rabbitries is a rigorous hygienic measure together with regular application of vaccination program **(Loliger and Eskens, 1991)**. Aluminum hydroxide gel is the most widely used adjuvant incorporated within the inactivated RHDV vaccine **(Arguello et al., 1989; Gunenkov et al., 1989; Kim et al., 1989; Arguello-Villares, 1991 and Salman, 2007)** and it is still used for production of inactivated RHDV vaccine in Egypt **(Daoud et al., 1998a and Salman, 2007)**. Inactivated RHDV W/O emulsion tissue vaccine was prepared either single **(Huang, 1991;Yu et al., 1992 and Salman, et al., 2009)** or combined with Pasteurella multocida **(Peshev and Christova 2003a and Taha et al., 2009)**. The type of adjuvant determines the duration of the immunity and protection that produced after vaccination which is longer for OE vaccines **(Pages, 1989)**. There is a great need to produce an inactivated vaccine having high adjuvant activity aiming to protect the rabbits for longer duration using one shot. SEPPIC, PUTEAUX CEDEX, France produces Montanide ISAs which are range of ready-to-use oil adjuvants recommended to be used for poultry and animal vaccines and rabbit is not found as targeted species. The raised question here is what is the most suitable type of Montanide ISAs to be used for production of inactivated RHDV vaccine? So, the present work was planned to prepare an inactivated RHDV oil emulsified vaccine

using different Montanide ISAs either W/O, W/O/W or W/P emulsions and evaluate its potency in vaccinated rabbits compared to Al $(OH)_3$ gel to detect the most suitable adjuvant for production of the vaccine.

Material and Methods

1. Material:

1-1- Rabbit Haemorrhagic Disease Virus (RHDV): Local Egyptian strain of RHDV designated as Giza/2006 **(Salman, 2007)** had HE963222 as accession no. in Gene Bank with a titer of $10^{6.61}$ LD_{50}/ml and of haemagglutination (HA) titer equal to 2^{14} HA unit was used for vaccine preparation, challenge of vaccinated rabbits and in haemagglutination inhibition (HI) tests.

1-2- Experimental rabbits: Three to four months old, industrial hybrid rabbits with an average body weight of 2 Kg were purchased from a conventional rabbitry without history of vaccination against RHDV. All rabbits were free of specific RHDV antibodies. The rabbits were used for RHDV propagation and vaccine evaluation.

1-3- Serum samples: Blood samples were collected from the experimental rabbits through the ear vein and allowed to coagulate then centrifuged in order to separate the serum. Sera of individual rabbits were subjected for inactivation process by heating in a water bath at 56°C for 15 minutes then kept in sterile vials at -20°C till examined serologically to measure the specific RHDV antibodies.

1-4- Positive and negative control serum of RHDV: It was supplied in Rabbit hemorrhagic disease viral antibody (RHDV-Ab) ELISA Kit from **Nova, Beijing, China.** It used in both ELISA and HI test.

1-5- Rabbit hemorrhagic disease viral antibody (RHDV-Ab) ELISA Kit: Cata. No. In-Rb0203, lot 201612. It used to assay RHDV-Ab levels in rabbit serum and it was supplied from **Nova, No. 18, Keyuan Road. DaXing Industry Zone, Beijing, China.**

1-6- Erythrocytes human type "O": The packed erythrocytes were suspended in sterile saline in a concentration of 0.75 % for micro-technique of HA and HI tests.

1-7- Adjuvants:

1-7-1- Rehydragel ®LV (CHEM TRADE): Aluminum hydroxide (Al $(OH)_3$) low viscosity gel. Stock No. 203120070602 was supplied by CHEM TRADE -BERKELEY HEIGHTS, NEW JERSEY. It was used according to manufacturer's instruction.

1-7-2- Montanide ISAs (SEPPIC, France) were supplied by SEPPIC, PUTEAUX CEDEX, France and used according to manufacturer's instruction.

1-7-2-1- Montanide ISA 70 VG Lot: T34651 was used at a ratio of 3:7 weight per weight (W/W)

1-7-2-2- Montanide ISA 71 VG Lot: T35031 was used at a ratio of 3:7 (W/W).

1-7-2-3- Montanide ISA 206 VG Lot: T34651 was used at a ratio of 1:1 (W/W).

Montanide ISA 760 VG Lot: U41921 was used at a ratio of 3:7 (W/W).

1-8- Chemicals:

1-8-1-Formaldehyde solution (Fluka Riedel-deHaen, Sigma, Germany): Lot No. 52930. 37% by weight stabilized with approximately 10% methanol was used for virus inactivation.

1-8-2- Sodium thiomersal (PARK scientific limited Northampton, UK): Lot No. P839F was prepared as a solution in a final concentration of 1/10000 weight per volume (W/V) and added to the prepared vaccine in a concentration of 1ml/ liter as a preservative.

2. Methods:

2-1 Haemagglutination (HA) test: Twofold dilutions of the RHDV suspension were incubated with an equal volume of washed human RBCs type "O" (0.75% concentration) in a V shaped-bottom micro-titer plate at 4°C according to **Capucci et al., (1996)** to determine HAU.

2-2 Haemagglutination inhibition (HI) test: It was carried out according to **Peshev and Christova, (2003a)** using 8 HA units of RHDV and human RBCs type "O" to estimate specific RHDV antibodies in rabbit sera. The antibody titer was the end-point of serum dilution showing inhibition of HA.

2-3 RHDV-Ab ELISA assay: The Microelisa strip plate provided in this kit has been pre-coated with an antigen specific to RHDV-Ab. The procedures were done according to protocol of the produced company **(Nova, Beijing, China).** Test effectiveness: the average value of positive control optical density (OD) ≥ 1.00; the average value of

negative control OD ≤ 0.10. The critical value (CUT OFF) calculation = the average value of negative control OD +0.15. Positive judgment when OD value ≥ CUT OFF. Negative judgment when OD value < CUT OFF.

2-4 Preparation of inactivated RHDV suspension: RHDV suspension incorporated into the vaccines was prepared according to **OIE, (2014)**. The viral inactivated suspension was assayed by HA test and it was found that RHDV antigen titer was 2^{10} HAU after inactivation as it is recorded by **Kim et al., (1989)**. Also **OIE, (2014)** recommended that HA titer of RHDV after inactivation for vaccine preparation should be higher than 2^7. Abolishing viral infectivity was carried out using formaldehyde at 0.4% concentration at 37°C for 48 hours. During the inactivation process, the fluid was continuously agitated.

2-5 Preparation of Al $(OH)_3$ inactivated RHDV vaccine: Inactivated RHDV vaccine with Al $(OH)_3$ adjuvant was prepared in a concentration of 20% Rehydragel volume per volume (V/V) according to **Khodeir and Daoud (2002)**.

2-6 Preparation of OE inactivated RHDV Vaccines: The oil emulsion vaccines were prepared, keeping the same antigen amount in the four vaccine formulations, as W/O for Montanide ISA 70 and 71, as W/P for Montanide ISA 760 and as W/O/W for Montanide ISA 206 according to manufacturer's instruction with different aqueous to oil ratio as follow, 30/70 (W/W) for Montanide ISA 70, 71 and 760 and 50/50 (W/W) for Montanide ISA 206. A preservative, thiomersal, was finally added at a dilution of 1/10,000 (V/V) before distribution into neutral glass of 10 ml capacity vials (each contains 5 ml vaccine). The vaccine was stored at 4°C till used.

2-7 Determination of the protective dose of the prepared vaccines:

It was carried out according to **Khodeir and Daoud (2002)**. Thirty five sero-negative rabbits were divided into 3 groups, 2 groups (1 and 2) each one contained 15 rabbits and the 3rd group (3) was test control contained 5 rabbits. The 1st two groups (1) and (2) were subdivided into 3 subgroups (a, b and c) each contained 5 rabbits. Group (1) used for Al $(OH)_3$ adjuvanted vaccine where subgroup (1a) inoculated with 0.5 ml S/C, subgroup (1b) inoculated with 1 ml S/C and subgroup (1c) inoculated with 1.5 ml S/C. Group (2) used for Montanide ISA adjuvanted vaccine where subgroup (2a) inoculated with 0.5 ml S/C, subgroup (2b) inoculated with 1 ml S/C and subgroup (2c) inoculated with 1.5 ml S/C. After 2 weeks, all inoculated and control rabbits were challenged I/M with 1ml of a virulent RHDV (10^3LD_{50}/ml- 2^{13} HAU) according to **Daoud et al., (1998a)**.

2-8 Quality control tests: The prepared vaccines were subjected to sterility and safety following standard international protocols of **British Pharmacopoeia Veterinary (2005)**.

2-8-1-Sterility test: The prepared vaccine was tested for the presence of viable bacteria, mycoplasma and fungi.

2-8-2-Safety: Safety test was carried out by S/C inoculation of five sero-negative rabbits with 3 times the vaccinal dose. The rabbits were observed for 3 weeks post inoculation for any notable signs of disease or local reaction.

2-9 Vaccine potency: Vaccine potency evaluation was based on antibody response measured by HI test and ELISA in addition to protection against challenge with virulent RHDV.

2-9-1-Experimental design: A total of 150 experimental 3 to 4 months old rabbits were housed in disinfected metal cages in a well ventilated and disinfected room receiving commercial pellet ration and clean water ad libitum. The rabbits were proved to be sero-negative for specific RHDV antibodies.

The rabbits were divided into 6 groups (25 rabbits for each). The 1st group (1) was vaccinated S/C with the prepared inactivated RHDV Al $(OH)_3$ gel adjuvanted vaccine in a dose of 0.5 ml per rabbit. The groups from 2 to 5 were vaccinated S/C with the prepared inactivated RHDV oil emulsion vaccines in a dose of 1 ml per rabbit, while the 6th group was kept unvaccinated negative control.

Group (1): vaccinated with Al $(OH)_3$ gel adjuvanted vaccine.

Group (2): vaccinated with Montanide ISA 70 adjuvanted vaccine.

Group (3): vaccinated with Montanide ISA 71 adjuvanted vaccine.

Group (4): vaccinated with Montanide ISA 206 adjuvanted vaccine.

Group (5): vaccinated with Montanide ISA 760 adjuvanted vaccine.
Group (6): unvaccinated
Each rabbit group was housed separately under well hygienic measure and kept under daily observation till the end of experiment.

2-9-1-Humoral immune response: It was followed up to 12 MPV for all groups starting from 0 time. Blood samples (individually from ear vein of rabbit) were collected weekly till the 4th WPV, every 2 weeks till the 8th WPV and then monthly till the 12th MPV. Sera were separated and kept at -20°C till used to evaluate humoral immune response through HI test and ELISA.

2-9-2- Challenge test: At the 3rd WPV and at the 6th MPV, randomly chosen 5 rabbits from each group either vaccinated (from 1st to 5th) or unvaccinated (6th) were transported to experimental isolators where they were challenged by intramuscular inoculation of 1ml of a suspension of virulent RHDV ($10^3 LD_{50}$/ml- 2^{13} HAU) **(Daoud et al., 1998a)**. The challenged rabbits were kept under daily observation for 2 weeks post challenge for any notable signs or deaths.

2-10 Statistical analysis: It was carried out up on the obtained data using ANOVA test according to **Sendecor (1971).**

Results

Four formulae of inactivated RHDV OE vaccines and one formula of inactivated RHDV Al $(OH)_3$ gel vaccine were obtained. The protective dose was found to be 0.5 ml and 1 ml inoculated S/C for both Al $(OH)_3$ adjuvanted and OE vaccines respectively providing 100% protection against the challenge with virulent RHDV (Table 1). The different formulae of prepared vaccines were proved to be sterile (no growth of micro-organisms on nutrient agar, blood agar and Sabaroud agar) and proved to be safe (The 5 inoculated rabbits S/C with three times the vaccinal dose did not show notable signs of disease or local reaction and remained healthy during the 3 weeks observation).

The humoral immunity was estimated using HI test and ELISA. Estimated mean specific RHDV HI antibodies were recorded and shown in table (2); none of the vaccinated and unvaccinated control rabbits had RHDV specific HI antibodies before vaccination. The mean titers for specific anti RHDV HI antibodies at 1st WPV for the vaccinated groups ranged from $2^{4.25}$ (in group (3) Montanide ISAs 71 adjuvanted vaccine) to $2^{6.6}$ (in group (5) Montanide ISA-760 adjuvanted vaccine). Mean titers for anti RHDV HI antibodies increased gradually in the different vaccinated groups reaching $2^{7.16}$ (in group (4) Montanide ISA 206 adjuvanted vaccine) to 2^9 (in group (5) Montanide ISA 760 adjuvanted vaccine) at 3rd WPV. At the 4thWPV these HI antibodies titers remained the same as 3rd WPV for groups (1) and (2), increased for group (4) and decreased for groups (3) and (5). The maximum level of mean RHDV HI antibody titer was attained at the 3rd MPV for all groups except groups (1) and (5) in which the maximum level was attained at 6th WPV and 4th MPV respectively. The highest titer between the different vaccinated groups was $2^{10.75}$ for group (2) (Montanide ISA-70), followed by titer of $2^{10.2}$ for group (4) (Montanide ISA-206) and this is the 1st peak. After that, it was noticed that the values of mean RHDV HI antibody titers of groups (2) to (5) went up and down throughout the monitoring period (corrugated line) giving more than one peak, while it went up then down (bell shape) in group (1).

The result of ELISA was recorded in table (3). According to the leaflet instruction of Elisa Kit, the critical value (CUT OFF) was calculated and found to be 0.25. Sera samples at pre-vaccination (0) time and that of group (6) all over the year gave ELISA mean OD less than CUT OFF value (0.25). The interpretation of ELISA mean OD values of other 5 vaccinated groups starting from 1st WPV showed that the vaccinated rabbits were considered immune or positive RHDV-antibody (ELISA mean OD was $\geq$ 0.25) throughout the year except group (1) which is positive for 10 months only.

All of the five formulae of RHDV vaccines resulted in 100% protection of vaccinated rabbits against challenge with virulent RHDV (10^3 LD_{50}/ml). This protection was at the 3rd WPV and at 6th MPV as shown in table (4).

Statistical analysis using ANOVA test revealed a significant difference (at $P \geq 0.05$) between the obtained anti-haemagglutinating antibody titers as well as ELISA mean OD in different vaccinated groups using different adjuvants.

Table (1) Protective dose of the prepared inactivated RHDV vaccines

Groups of rabbits		The tested dose	No. of vaccinated rabbits	No. of challenged rabbits	No. of protected rabbits	Protection percent
*Group (1)	A	0.5 ml	5	5	5	100%
	B	1 ml	5	5	5	100%
	C	1.5 ml	5	5	5	100%
**Group (2)	A	0.5 ml	5	5	3	60%
	B	1 ml	5	5	5	100%
	C	1.5 ml	5	5	5	100%
***Group (3)		-	-	5	0	0%

*Group (1) = received Al $(OH)_3$ gel vaccine- **Group (2) = received oil emulsion vaccine-***Group (3) = unvaccinated control.

Table (2) Mean of specific RHDV HI antibody titers (log2) in sera of vaccinated and unvaccinated rabbits

Time post vaccination	Geometric mean of RHDV specific HI antibody titers (log2)					
	Group(1)	Group(2)	Group(3)	Group(4)	Group(5)	Group(6)
0 day	0	0	0	0	0	0
1st WPV	6	5	4.25	5.4	6.6	1
2nd WPV	6.4	7.9	5.2	6.5	7.4	2
3rd WPV	7.6	7.8	7.64	7.16	9	1
4th WPV	7.6	7.8	6.2	9	7.6	1
6th WPV	8.33	8.8	6.2	7.8	9.8	0
2nd MPV	8.23	8.5	8.8	8.5	7.8	2
3rd MPV	8.2	10.75	10	10.2	7	1
4th MPV	6.33	8.3	8	8.66	10.11	1
5th MPV	6.2	10	7.66	8.9	7.3	1
6th MPV	6.5	9.2	8.5	10	7.8	0
7th MPV	6	10.4	9.7	10	9	2
8th MPV	6	9.6	8.8	9.2	9	2
9th MPV	5.6	10	9.5	9.5	9.5	0
10th MPV	5.2	10.2	9	9.5	8	1
11th MPV	4	9	8	8.6	8.5	1
12th MPV	3	10	8	9.7	7.6	1

WPV= Week Post Vaccination. MPV= Month Post Vaccination

Group (1): vaccinated with Al (OH)3 gel adjuvanted vaccine. Group (2): vaccinated with Montanide ISA 70 adjuvanted vaccine. Group (3): vaccinated with Montanide ISA 71 adjuvanted vaccine. Group (4): vaccinated with Montanide ISA 206 adjuvanted vaccine. Group (5): vaccinated with Montanide ISA 760 adjuvanted vaccine. Group (6): unvaccinated

Antibody titers in control group were non specific and non protective.

Table (3) ELISA mean optical density (OD) for detection of RHDV antibody titers in sera of vaccinated and unvaccinated rabbits

Time post vaccination	Mean of OD at 450 *nm					
	Group (1)	Group (2)	Group (3)	Group (4)	Group (5)	Group (6)
0 day	0.241 -ve	0.221 -ve	0.205 -ve	0.243 -ve	0.198 -ve	0.206 -ve
1st WPV	0.365 +ve	0.292 +ve	0.269 +ve	0.339 +ve	0.382 +ve	0.217 -ve
2nd WPV	0.389 +ve	0.491 +ve	0.354 +ve	0.382 +ve	0.417 +ve	0.245 -ve
3rd WPV	0.455 +ve	0.473 +ve	0.479 +ve	0.432 +ve	0.527 +ve	0.204 -ve
4th WPV	0.473 +ve	0.459 +ve	0.365 +ve	0.517 +ve	0.455 +ve	0.244 -ve
6th WPV	0.500 +ve	0.543 +ve	0.347 +ve	0.454 +ve	0.576 +ve	0.246 -ve
2nd MPV	0.499 +ve	0.506 +ve	0.534 +ve	0.505 +ve	0.488 +ve	0.249 -ve
3rd MPV	0.503 +ve	0.611 +ve	0.576 +ve	0.587 +ve	0.402 +ve	0.245 -ve
4th MPV	0.363 +ve	0.5055 +ve	0.5 +ve	0.526 +ve	0.575 +ve	0.249 -ve
5th MPV	0.365 +ve	0.585 +ve	0.443 +ve	0.413 +ve	0.421 +ve	0.245 -ve
6th MPV	0.358 +ve	0.533 +ve	0.553 +ve	0.555 +ve	0.450 +ve	0.215 -ve
7th MPV	0.345 +ve	0.586 +ve	0.576 +ve	0.582 +ve	0.500 +ve	0.240 -ve
8th MPV	0.349 +ve	0.544 +ve	0.528 +ve	0.563 +ve	0.521 +ve	0.240 -ve
9th MPV	0.330 +ve	0.590 +ve	0.568 +ve	0.566 +ve	0.566 +ve	0.199 -ve
10th MPV	0.283 +ve	0.567 +ve	0.508 +ve	0.568 +ve	0.459 +ve	0.242 -ve
11th MPV	0.237 -ve	0.546 +ve	0.475 +ve	0.513 +ve	0.524 +ve	0.234 -ve
12th MPV	0.245 -ve	0.556 +ve	0.459 +ve	0.548 +ve	0.434 +ve	0.205 -ve

WPV= Week Post Vaccination. MPV= Month Post Vaccination

Group (1): vaccinated with Al (OH)3 gel adjuvanted vaccine. Group (2): vaccinated with Montanide ISA 70 adjuvanted vaccine. Group (3): vaccinated with Montanide ISA 71 adjuvanted vaccine. Group (4): vaccinated with Montanide ISA 206 adjuvanted vaccine. Group (5): vaccinated with Montanide ISA 760 adjuvanted vaccine. Group (6): unvaccinated

OD = optical density. According to kit instructions, OD measured spectrophotometrically at a wave length of 450 nm, OD value≥ 0.25 considered RHDV- antibodies positive and OD < 0.25 considered RHDV- antibody negative. nm= nanometer

Table (4): Potency of different inactivated RHDV vaccines with four types of Montanide adjuvants and aluminum hydroxide gel adjuvant

Groups of rabbits	Vaccination type	3rd WPV*				6th MPV*			
		Number of challenged rabbits	Number of protected rabbits	Number of dead rabbits	Protection percent	Number of challenged rabbits	Number of protected rabbits	Number of dead rabbits	Protection percent
Group 1	Al $(OH)_3$ gel adjuvanted vaccine	5	5	0	100%	5	5	0	100%
Group 2	Montanide ISA 70 adjuvanted vaccine	5	5	0	100%	5	5	0	100%
Group 3	Montanide ISA 71 adjuvanted vaccine	5	5	0	100%	5	5	0	100%
Group 4	Montanide ISA 206 adjuvanted vaccine	5	5	0	100%	5	5	0	100%
Group 5	Montanide ISA 760 adjuvanted vaccine	5	5	0	100%	5	5	0	100%
Group 6	Unvaccinated	5	0	5	0%	5	0	5	0%

WPV = week post vaccination. MPV= month post vaccination.

Discussion

RVHD is the major viral disease affecting the European rabbits (Oryctolagus cuniculus) and responsible for high economical losses in rabbitries and high mortality rate in wild rabbit population **(Barcena et al., 2000)**.Vaccination is proved to be the most successful preventive and control measure against RHDV even during sever outbreak at which restocking of rabbit colonies during outbreak without vaccination is unsuccessful **(Kpodekon and Alogninouwa, 1998 and El- Khashab et al., 2001)**. RVHD became endemic in Egypt where it was recorded in different Egyptian provinces **(El-Zanaty, 1994; Abd El-Ghaffar et al., 2000 and Salman, 2007)**. RVHD was controlled successfully using inactivated RHDV tissue vaccine **(Liu et al., 1984; Kim et al., 1989 and Smid et al., 1991)**. In Egypt, RVHD control depends on the wide use of inactivated RHDV vaccine either local (with Al $(OH)_3$ gel adjuvant) or imported from Spain (with oil adjuvant CUNIPRAVAC-RHD) **(Salman, 2007 and Taha et al., 2009)**. Aluminum hydroxide gel adjuvant is still used for production of inactivated RHDV vaccine in Egypt in VSVRI according to method described before by **Daoud et al., (1998a); Salman, (1999) and Salman, (2007)** in spite of oil emulsified vaccine was prepared using paraffin oil locally **(Salman, 2009)** in addition to combined bivalent oil emulsified vaccines against RVHD and rabbit Pasteurellosis was prepared using Montanide ISAs **(Taha et al., 2009)**. Immunity to RHDV after vaccination is lasting at least 6 months but OE tissue vaccine has longer lasting potency **(Huang, 1991)**. So, the objective of this study was to detect the most suitable Montanide ISA for production of an inactivated RHDV oil emulsified vaccine. Montanide ISAs consists of a series of adjuvants composed of a variety of oils, emulsions characteristics, emulsifiers and immunomodulators and they are known to be used in production of different oil emulsion veterinary vaccines owing to their reputation in enhancing the immune response **(Mark et al., 2012)**.

Montanide ISAs 70 and 71 are W/O emulsions achieve long-term protective immune responses; while Montanide ISA 760 is W/P induces strong and long term immunity. Montanide ISA 206 is W/O/W emulsion induces both short- and long-term protective immune responses. Two formulae of vaccines with Montanide ISA 70 and 71 were prepared as the other W/O vaccines consisted of an aqueous phase suspended as droplets in mineral oil; the RHDV antigen was contained in aqueous phase and remained dispersed in the oil or suspending phase through the action of emulsifiers as stated by **Stone et al., (1983)**. Vaccine with Montanide ISA 760 was prepared as W/P emulsion and vaccine with

Montanide ISA 206 was prepared as W/O/W emulsion while RHDV antigen was contained adsorbed on Al $(OH)_3$ gel particles in the formula of vaccine with Al $(OH)_3$ gel **(Rajesh and Rost 2000)**. Inactivated RHDV content in the vaccine was 2^{10} HAU in accordance with **Kim et al., (1989)** and more than 2^7 as recommended by **OIE, (2014)**. **Huang, 1991 and Smid et al., 1991** reported that immunity to RHDV after vaccination is rapidly developed in the vaccinated rabbits and persisted for more than 6 months. In our study, the specific anti RHDV HI antibodies began to be detected from the 1st WPV in agreement with **Wei et al., (1987); Haralambiev et al., (1990); Popovic, (1990) and Smid et al., (1991)**. Both titers of groups (2) and (3) were the lowest owing to their formula as W/O emulsions, while the highest titer at 1st WPV was for group (5) also owing to its formula as W/P emulsion which induces strong immunity. Al $(OH)_3$ gel and Montanide ISA 206 adjuvanted vaccines gave moderate titers in group (1) and (4) explained by watery phase inducing fast immune response. This result agreed with those obtained by **Peshev and Christova (2003b)** who used RHDV oil adjuvanted vaccine too and obtained $2^{6.12}$ HI antibodies at 1st WPV. Also **Taha et al., (2009)** recorded HI antibodies values ranged from 2^6-$2^{7.25}$ at 1st WPV which is nearly equal to our results and he used Montanide ISA-50 and the vaccine was RHDV combined with P. multocida. The higher antibody titer following vaccination with oil emulsified vaccine was attributed to low viscosity and high homogenicity as stated before by **Gomes et al., (1980)**, while the obtained titer of group (1) at 1st WPV (2^6) was lower than that obtained by **Daoud et al., (1998a)** ($2^{8.2}$) in spite of using the same adjuvant. At the 4thWPV these HI antibodies titers remained the same as 3rd WPV for groups (1) and (2), increased for group (4) and decreased for groups (3) and (5) and it was found that these HI antibody titers were within the range of other titers recorded at the same interval post vaccination by **Shevchenko, (1994)** who recorded $2^{8.164}$ HI antibodies and **Daoud et al., (1998a)** who recorded $2^{8.8}$ HI antibodies in spite of difference in the used adjuvant.

The highest titer between the different vaccinated groups was $2^{10.75}$ for group (2) (Montanide ISA-70) in agreement with **Taha et al., (2009)** who used the same adjuvant, followed by titer of $2^{10.2}$ for group (4) (Montanide ISA-206) and this is the 1st peak. After that, it was noticed that the values of mean titers of HI RHDV-antibodies of groups (2) to (5) went up and down throughout the monitoring period giving more than one peak, while it went up then down in group (1). These results were attributed to the nature of adjuvant incorporated within the vaccine, where OE vaccines gave and elicited immune response run in a zigzag like manner as stated before by **Thayer et al., (1983)**. Also **Mohi-ud-din et al., (2014)** said that Montanide ISA 206 VG produces double-emulsion vaccine very fluid, stable, well tolerated and induces short- and long-term immune response.

The ELISA mean OD values for vaccinated groups went parallel to HI antibody titers as shown in table (3) confirmed the results of humoral immune response measured in HI test. **Smid et al., (1991) and Daoud et al., (1998a)** followed the immune response of vaccinated rabbits using ELISA too and recorded ELISA mean OD but till 28th day post-vaccination.

By statistical analysis using ANOVA test it was found that there was a significant difference (at $P \geq 0.05$) between the obtained anti-haemagglutinating antibody titers as well as ELISA OD in different vaccinated groups being more prominent for group (2) followed by group (4).

In conclusion, Montanide ISAs adjuvanted vaccines gave higher antibody titers than Al $(OH)_3$ gel vaccine and for longer time extended all over the year especially for groups (2) and (4) which ended with 2^{10} and $2^{9.7}$ HI antibody titer, 0.556 and 0.548 ELISA mean OD at 12th MPV and this could be attributed to oil adjuvant vaccine antigen which is slowly released from depot. This in agreement with **Elham and Hoda (2011) and Mohi-ud-din et al., (2014)** that used Montanide ISA-50 and 206 but with P. multocida in rabbits. Also our results agreed

with **Huang (1991)** who reviewed that OE vaccine induced higher HI antibody titers and duration longer than Al $(OH)_3$ gel in RHDV vaccine.

The challenge resulted in 100% protection and this is identical with that recorded by **Shevchenko, (1994)** who showed that RHDV vaccine resulted in100% protection of rabbits, **Salman, (1999)** who found that the protection percentage against the challenge with $10^{3.59}$ LD_{50} of RHDV was 100% in the vaccinated rabbits. Also the challenge result agree with the result of **Smid et al., (1991) and Daoud et al., (1998a)** who recorded that rabbits developed full protection against RHDV infection 3 weeks after the administration of a single dose of inactivated RHDV vaccine. Protection against clinical disease was expected with the specific RHDV HI antibody titer, induced in vaccinated rabbits, and proved that all the prepared inactivated RHDV vaccines having sufficient amount of RHDV antigen that may have the potential to induce higher level of protection against infection than is currently realized, this result comes in contact with those of **Stone et al., (1983).** Our results also agreed with those of **Nowotny et al., (1993)** who found that the adult rabbits with RHDV-antibody titers ranging from 2^6 to 2^{13} remained clinically healthy after inoculation with virulent RHDV and **Simon et al., (1993)** who concluded that a titer> 20 HIU was protective.

From the aforementioned results, it could be concluded that all Montanide ISAs used in this study could be used to produce inactivated RHDV OE vaccine and it was found to be more preferable than Al $(OH)_3$ gel for long term immunity and regarding statistical analysis, Montanide ISA 70 found to be the most suitable Montanide ISA followed by Montanide ISA 206 then Montanide ISA 760 and finally Montanide ISA 71 and could be used safely for active immunization of rabbits against this disease that threaten rabbit industry in Egypt. Also it could be concluded that a program of vaccination against RHDV could be applied by using more than one type of the prepared vaccines for example it could be start with Al $(OH)_3$ gel adjuvanted vaccine for fast response then boosted with any of the other 4 formulae of oil emulsion vaccines for long term immunity.

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الملخص العربي

استخدام أنواع مختلفة من المونتانيد في تحضير لقاح النزف الدموي الفيروسي الأرنبي المثبط

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مرض النزف الدموي الأرنبي ذو أهمية اقتصادية كبيرة بسبب النفوق العالي الذي يحدثه في الأرانب البالغة، في هذه الدراسة تم تحضير أربعة تراكيب زيتية من لقاح فيروس مرض النزف الدموي الأرنبي المثبط باستخدام اربعة أنواع مختلفة من مادة المونتانيد لشركة سيبيك الفرنسية (مونتانيد 70 و71 و206 و 760) و تم تحضير تركيبة اخرى باستخدام مادة جل هيدروكسيد الألومنيوم. وجد أن الجرعة الكافية لحماية الأرانب من اللقاحات الزيتية هي 1 مل و من لقاح هيروكسيد الألومنيوم هي 0.5مل، تم دراسة تأثير كفاءة هذه اللقاحات فى الأرانب المحصنة لمدة سنة كاملة بعد التحصين من حيث الاستجابة المناعيه و مقاومة التحدي بفيروس النزف الدموي الأرنبي الضاري حيث تم حقن 5 مجموعات من الأرانب الخالية من الأجسام المضادة الخاصة بهذا المرض باللقاح مع وجود مجموعة اخرى من الأرانب غير محصنة كضابط للتجربة، أثبتت كل اللقاحات المحضرة كفاءة مناعية عالية حيث بدأت الأجسام المضادة في الظهور من الأسبوع الأول بعد التحصين، ارتفع مستوى الأجسام المضادة المقاسة باختبار منع التلازن الدموي و الأليزا في الأرانب المحصنة حيث بلغ أعلى مستوى له في الشهر الثالث بعد التحصين في المجموعات من الثانية حتى الرابعة أما المجموعتين الأولى و الخامسة فكان أعلى مستوى لهما في الأسبوع السادس و الشهر الخامس بعد التحصين على الترتيب، استمر مستوى الأجسام المضادة مرتفعا حتي نهاية السنة في اللقاحات الأربعة المستخدم فيها مادة المونتانيد في حين أن اللقاح المستخدم به جل هيدروكسيد الألومنيوم اخذ هذا المستوى في الأنخفاض بعد الشهر السادس، عند اجراء اختبارالتحدي في الأسبوع الثالث بعد التحصين و كذا الشهر السادس بعد التحصين كانت نسبة الحمايه ضد فيروس النزف الدموي الأرنبي الضاري 100% لكل المجموعات المحصنة و 0% للمجموعة الغير محصنة، من هذه النتائج يمكن التوصية باستخدام زيوت المونتانيد لكونها أفضل من جل هيدروكسيد الألومنيوم في تصنيع لقاح فيروس مرض النزف الدموي الأرنبي و بأفضلية مرتبة كما يلي، مونتانيد 70 ثم 206 متبوعا بمونتانيد 760 و في النهاية مونتانيد 70 لما لهم من تأثير في احداث مستوى مناعي أعلى و لفترة أطول مما يوفر العملة الصعبة المهدرة في استيراد هذا اللقاح من الخارج.