



Evaluation of The Cellular and Humoral Immune Response of Sheep Vaccinated with Inactivated Rift Valley Fever Vaccine Adjuvanted with Montanide Gel 01™

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ABSTRACT

Rift Valley Fever (RVF) is an acute infectious zoonotic arthropod - born viral disease, affecting many species of animals with causing great economic losses in animal wealth. Vaccination of susceptible animals with RVF vaccines are an important factor for controlling the disease. This study was applied to improve the locally produced vaccine by using Montanide Gel 01™ as an adjuvant in comparison with Aluminium Hydroxide inactivated RVF vaccine, through evaluating the humeral, cellular immune response and the duration of immunity in sheep vaccinated with the prepared vaccines. The antibody titre in the group vaccinated with one dose of Montanide Gel 01™ inactivated RVF vaccine reached the protective level at the 7th-day post-vaccination and stayed within the protective level till the end of the 11th month, while in the group vaccinated with 2 doses, the antibody titre stayed within the protective level till the end of 13th month in sheep vaccinated with the same vaccine. These results revealed that the best vaccine is 20% Montanide Gel 01™ inactivated RVF vaccine (2 doses) as it gave a higher level of antibody throughout the experiment compared with that of other vaccinated groups. Also, the results of cell-mediated immune response showed that: (i) The cell proliferation expressed by optical density showed early significance in sheep vaccinated by Montanide Gel 01™ inactivated RVF vaccine and a slight elevation in sheep vaccinated by Aluminium Hydroxide inactivated RVF vaccine. (ii) The phagocytic activity expressed by phagocytic percentage and phagocytic index showed early significant in sheep vaccinated with Montanide Gel 01™ inactivated RVF vaccine and a slight elevation in sheep vaccinated by Aluminium hydroxide inactivated RVF vaccine. Also, high levels of IL-6 and IL-12 gene expression were detected in sheep vaccinated with Montanide Gel 01™ inactivated RVF vaccine as shown by RT-PCR. All the previous data showed that the Montanide Gel 01™ was highly immunogenic than Aluminium Hydroxide beside that Montanide Gel 01™ inactivated RVF vaccine induces rapid onset immunological response with a long duration which recommended for emergency vaccination.

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INTRODUCTION

Rift Valley Fever (RVF) is a viral disease transmitted by mosquitoes. The disease appears as epizootics in domestic cattle and sheep, producing widespread abortions and deaths in young animals. It is a zoonotic disease and highly infectious in Humans, (Digoutte and Peters, 1989). The disease occurs in climatic conditions combined with rainfall every 5-25

years in nonimmune animal's populations (OIE, 2014). RVF disease is caused by Bunyavirus of the Genus Phlebovirus and transmitted by mosquitoes (OIE, 1989).

The best tool for protecting the animal population and indirectly humane being is vector control and vaccination, (Abdel-Gaffar *et al.*, 1979), using a safe and potent inactivated vaccine. During

epizootics of RVF, the use of live attenuated Smithburn vaccine is recommended (WHO, 1983) but with limitation to be used in pregnant animals due to fear from teratogenic or abortogenic effect and ability to be converted into a virulent state (Kathryn *et al.*, 1991). The progress in vaccine production is directed towards the selection of the proper adjuvant that can elaborate high and long-standing immunity. Adjuvants are considered as one of the important factors in vaccine formulations that increase the immune response either humoral and cell-mediated immunity (Dalsgrd *et al.*, 1990).

Montanide Gel 01TM is generic of Montanide as water-based adjuvant designed recently to improve the efficacy of aqueous type vaccine. It was observed that using of Montanide Gel 01TM as adjuvant gave sufficient early immune response in vaccinated cattle associated with a perfect safety profile whatever the nature of the antigen (Dupuis *et al.*, 2008), and it was reported that the antibodies induced were higher than Aluminium-based vaccines and so it can be used associated with a wide range of antigenic media and recommended to be used as adjuvant for sensitive animal's vaccines (Parker *et al.*, 2009) and (Deville *et al.*, 2011).

This work aims to improve and enhance the immunogenicity of the locally produced inactivated RVF vaccine to induce high and long duration of immunity in vaccinated animals using Montanide Gel 01TM as an adjuvant.

MATERIALS AND METHODS

A. MATERIALS

1. Experimental animals

Sheep

Twenty-five local breed sheep 3-4-month-old were used for evaluation of their immune response to the prepared vaccines. All of these animals were screened using SNT and ELISA and proved to be free from RVF antibodies. They were housed under strict hygienic measures in insect-proof stables receiving balanced ration and adequate water. Also, these animals were examined and proved to be healthy and free from external and internal parasites.

Adult mice

Swiss albino weaned mice and specific pathogen-free, 21-30 days old supplied by the breeding unit, Veterinary Serum and Vaccine Research Institute, Abbasia, Cairo, Egypt were used for toxicity test of different concentrations of Montanide Gel 01TM, titration of the virus, and evaluation of the immune response of the prepared vaccines.

2. Tissue Culture Cells

Baby Hamster Kidney cells (BHK21), were grown and maintained according to Macpherson and stocker (1962), and were used for propagation and titration of RVF virus, vaccine production.

3. Virus

The original Rift Valley Fever (RVF) virus was isolated from a human patient in Zigzag, Sharkia Province, Egypt and supplied by NUMRU -3. It was designated as ZH501 with a titer of $10^{7.5}$ TCID₅₀ / ml.

4. Samples

Blood samples

Serum samples were collected from sheep before and after vaccination with the prepared vaccines. Sera were stored at -20°C and inactivated at 56°C for 30 minutes before being used in the serological studies (SNT and ELISA).

Heparinized blood samples

Jugular blood samples from vaccinated and non-vaccinated sheep were collected with anticoagulant (heparin 20-40 IU/ ml) for evaluation of cell-mediated immune response by lymphocyte blastogenesis assay, phagocytic activity test and measuring of interleukin 6 and 12.

5. Reagents, kits and media

5.1. Adjuvants:

Aluminium hydroxide gel: The gel was obtained from (Alliance Bio Company, USA), Lot. No. 11-274-30 was used in a concentration of 20% for local vaccine preparation.

Montanide Gel 01TM: Montanide Gel 01TM is based on synthetic polymer classified in the category of high molecular weight polyacrylic acid. It is a white, opaque and flowable gel. Its characteristic viscosity and its ability to work under all pH conditions make it very easy to handle. It is available in preserved or sterile autoclaved grades. It elicits humoral and cell-mediated immune response without mortality, toxicity or pyrogenic effect, simple to formulate, produced stable emulsion with stability at least 1 year at 4°C. It was obtained from Seppic, Paris, France.

5.2. RVF antigen: RVF cell lysate antigen was used for IgG antibody detection using ELISA technique. It was Kindly Supplied by RVF department, veterinary serum and vaccine research institute (VSVRI), Abbasia, Cairo.

5.3. Positive control serum: Positive RVF serum was kindly obtained from RVF department Serum and Vaccine Research Institute, Abbasia, Cairo.

5.4. Negative control serum: Serum from susceptible sheep tested and proved to be free from antibodies against RVF virus.

5.5. ELISA kits: Cat. No. 3340, was obtained from COSTER 205 Broadway Cambridge, MA 02139.

5.6. Candida albicans: It was kindly supplied by the department of microbiology. The central lab for quality control veterinary biological products, it was used in macrophage activity test.

5.7. Fycoll-hypaque: (Lymphocyte separation medium), used for the separation of mononuclear leukocyte cells from peripheral blood.

5.8. Roswer Park Memorial Institute (RPMI-1640) Medium: RPMI-1640 tissue culture medium with L-glutamine, sodium bicarbonate and antibiotics, was obtained from Flow Lab, UK. Medium sterilization by filtration through a Millipore filter (0.22 µm, Sartorius, Germany) followed by storage at 4°C until used in lymphocyte blastogenesis assay and phagocytic activity test.

5.9. Methyl alcohol: It is Methanol free from acetone (99.7%). Country of origin is Germany, from Sigma Company. It was used as 0.3 cm/well for macrophage activity test.

5.10. Giemsa stain: It was used for phagocyte staining blood sample.

5.11. Fetal bovine serum: supplied by GIBCO, limited, U.K. used for separation of blood lymphocytes.

5.12. Cell proliferation kit (Mdbiosciences-USA): The kit was used in the lymphocyte blastogenesis assay Cat #: 409005.

5.13. Regents for total RNA extraction from mice lymphocytes using

Trizol:

- Trizol reagent (Invitrogen, Carlsbad, CA).
- Phenol crystals.
- Tris base.
- Distilled water.
- Concentrated HCL to adjust PH of Tris HCL.
- Chloroform.
- Isopropyl alcohol.
- 75% ethanol in RNA free water.
- RNA free water.

5.14. Regents and kits for 1st strand cDNA synthesis:

- Anchored oligo (dT) 18 primer.
- Transcriptor 1st strand cDNA synthesis kit (Roche,) using Access

Quick RT-PCR (Promega, USA) Cat. No. A1702 of 100 reactions.

5.15. Regents and kits for Syber green quantitative real-time PCR:

Primers:

IL 6 gene Primers According to **Ebrahim et al., (1995)**

IL 12 gene primers according to **Sangeeta et al., (2006)**

Cytokine	Primer sequence	SIZE
IL 6 F.	5'-ACTTCATCCGAATAGCTCTC-3'	624 bp
IL 6 R.	5'-ATGAACTCCCTCTTCACAAAG-3'	
IL12 F.	5'-TCTCGGCAGGTGGAAGTCA-3'	144 bp
IL12 R.	5'-ACTTTGGCTGAGGTTTGGTCTG-3'	

SYBER Green qPCR kit

Maxima™ SYBR Green qPCR Master Mix (2X) and ROX Solution (fermentas,) cat no. #K0259 for 60 reactions of 25 µl.

Kit contents

1- 2XMaxima™ SYBR Green qPCR Master Mix and a ready-to-use solution containing:(Maxima™ Hot StartTaq DNA Polymerase, SYBR® Green dye I and dNTPs in an optimized PCR buffer).

2- ROX solution as a passive reference dye

3- RNase-Free Water

Real-time PCR machine: Stratagen MX3005P machine (Stratagen USA).

METHODS

1. Toxicity test of different concentrations of Montanide Gel 01™ Adjuvant in adult mice

Different concentrations of Montanide Gel 01™ adjuvant in media were prepared (10%, 15%, 20 and 25%), for each concentration 10 mice were inoculated (I/P 0.2 ml/mice) and kept under daily observation for 10 days post-inoculation (**OIE 2012**).

2. Adsorption test

To detect the most suitable concentration of the adjuvant that can adsorb the efficient amount of antigen by adding each concentration of Montanide Gel 01™ to known virus titre 10^7 TCID₅₀ / 0.1 ml and then centrifuge the mixture at 1500 rpm for 15 minutes, then titrate the virus remained in the supernatant fluid.

3. Inactivation process: using BEI according to **Blackburn and Besselaar (1991)**.

4. Checking the inactivation process

Samples from the inactivated virus were checked for the presence of active virus as follows:

Safety in Tissue culture: according to **OIE (2012)**.

Safety in baby mice: according to **El Nimr (1980) and Eman (1995)**.

5. Preparation of RVF vaccine batches

Preparation of 2 batches of inactivated RVF vaccine as follow:

Batch 1: using Aluminium hydroxide gel 20% concentration as an adjuvant.

Batch 2: using Mmontanide gel 20% concentration as an adjuvant.

6. Evaluation of the prepared vaccine batches

6.1. Sterility test: The prepared vaccines were tested for their freedom from Mycoplasma, aerobic, and anaerobic bacteria, fungi.

6.2. Potency test: Fivefold dilutions of each vaccine were prepared in suitable media starting from 1:1 to 1:625. Five groups of adult mice. (21-28 days old) were used for each dilution and each mouse was inoculated with two doses 0.2 ml of the vaccine I/P, one week apart. Seven days after the second inoculation, all animals were challenged via I/P route with 0.1 ml of RVF virus containing 10^4 MIPD₅₀ / ml. In addition to the other two groups of mice, one group was inoculated with challenge virus as positive control and the other group was kept as a non-vaccinated non-challenged negative control (OIE, 2012).

All groups of mice were kept under observation for 21 days and deaths were recorded daily. The ED₅₀ / ml was calculated according to the method of **Reed and Muench, (1938)**. Deaths occurring during the first day were considered non-specific.

7. Sheep vaccination

Twenty five sheep of 3 – 4 months old were kept in an isolated place and were divided into 5 groups (5 animals/group) as follows:-

Group 1: Each animal vaccinated (S/C) with 1ml of inactivated aluminium hydroxide gel adjuvanted RVF vaccine as one dose.

Group 2: Each animal vaccinated (S/C) with 1ml of inactivated aluminium hydroxide gel adjuvanted RVF vaccine in two doses

Group 3: Each animal vaccinated (S/C) with 1ml of inactivated 20% Montanide Gel 01™ adjuvanted RVF vaccine as one dose.

Group 4: Each animal vaccinated (S/C) with 1ml of inactivated 20% Montanide Gel 01™ adjuvanted RVF vaccine in two doses.

Group 5: kept as non-vaccinated (control negative).

8. Collection of Samples

• **For evaluation of the humoral immune response:** Sera samples were collected from all sheep before and after vaccination at intervals of 7, 14, 28 days and then monthly for 12 months. (Samples were collected in sterile dry clean screw-capped bottles, then left at room temperature for 1-2 hours or incubated for 30 minutes then placed in refrigerator overnight, then serum was removed by using sterile pasture pipette and centrifuged at 3000 rpm for 10 minutes), then kept at -20°C until used for antibody detection.

• **For cell-mediated immune response:** blood samples collected on heparin with intervals of 1,3,5,7,10 and 15 days post-vaccination.

9. Evaluation of the humoral immune response

9.1. Serum Neutralization Test (SNT): This test was used to detect the specific neutralizing antibodies against RVFV in the serum samples of vaccinated sheep according to the method of constant serum- virus dilution procedure. The serum-neutralizing index was calculated according to **Reed and Muench, (1938)**.

9.2. Indirect Enzyme-Linked Immunosorbent Assay (ELISA): According to (**Voller et al., 1976**), indirect method of ELISA. The cut off value is calculated according to **Eduard, (1985)**.

Cut off = mean optical density of negative control samples + 3 (SD of mean negative control samples).

10. Evaluation of cell-mediated immune response

10.1. Evaluation of cell-mediated immune response by lymphocyte blastogenesis using XTT assay:

The cleavage of the tetrazolium salt (3-[4, 5-dimethyl thiazole-2yl]-2,5 -diphenyl tetrazolium bromide) into a blue coloured product (formazan) by the mitochondrial enzyme succinate dehydrogenase (**Slater et al., 1963**) is very useful for assaying the cell survival and proliferation. This conversion takes place in the living cells and the amount of formazan produced is proportional to the number of cells present.

The assay was performed as follow:

i) **Separation of lymphocytes:** Separation of lymphocytes was applied according to **Lucy, (1977)** and **Lee, (1984)**.

ii) **Setting up of lymphocyte and using cell proliferation kit (XTT kit):** The test was conducted according to **Scudiero et al., (1988)**.

10.2. Evaluation of the phagocytic activity of macrophages by using Candida Albicans:

i) **Separation and cultivation of mononuclear cells:** According to **Aronthey and Hazen (1988)**.

ii) **Evaluation of phagocytic activity:**

Phagocytic %: It was performed by the method of **Harmon and Glisson, (1989)**, which was modified by **Hussein, (1989)**

$$\text{Phagocytic \%} = \frac{\text{No. of phagocytes ingest candida}}{\text{Total No. of phagocytes}} \times 100$$

Phagocytic index: It was done according to **Richardson and Smith, (1981):**

$$\text{Phagocytic index} = \frac{\text{Total No. of phagocytes ingest more than 2 candida}}{\text{Total No. of phagocytes ingest candida}}$$

10.3. (IL6 and IL12) gene analysis by SYBER Green quantitative real time PCR (qPCR) for sheep:

10.3.1. Separation of sheep blood lymphocytes according to **Lucy, (1977)** and **Lee, (1984)**.

10.3.2. Total RNA extraction from sheep lymphocytes using Trizol reagent (Invitrogen, Carlsbad, CA): This solution was stored at room temperature and was diluted 1:10 with distilled water before use and pH were adjusted.

10.3.3. 1st strand cDNA synthesis: Reaction mix volumes/ one reaction using 1st strand cDNA synthesis by Access quick RT-PCR.

Component	Amount
Access quick™ master mix, 2x	15 µl
Uni 12 primer	10 µl
Nuclease free water	0.4 µl
AMV reverse transcriptase (RT-enzyme)	0.6 µl
RNA template	4 µl
Final volume	30 µl

Thermal profile:

Steps	Temperature/Time
Reverse transcription	45°C/45 minutes
AMV RT inactivation and RNA/cDNA/primer denaturati	92°C/5 minutes
Hold (refrigeration)	4°C/∞

10.3.4. Syber green real-time PCR For quantification of IL 6 and IL12 in lymphocytes

Reaction component	Volume/reaction
Maxima™ SYBR Green qPCR master mix (2X)	12.5 µl
Forward primer	1 µl (10 pmol)
Reverse primer	1 µl (10 pmol)
Template DNA	(500 ng)
Nuclease free water	To 25 µl

The volume of cDNA added from the RT reaction should not exceed 10% of the final PCR volume.

Thermal profile:

Step	Temp./time
Initial denaturation.	95°C/3 min
Denaturation	95°C/30 sec.
Annealing	55°C/1 min.
Extention	72°C/1 min
No. of cycles (40) cycles	
Dissociation curve	95°C/1 min. 55°C/30 sec. 95°C/30 sec.

11.Statistical analysis:

The obtained data were statistically analysed by (SPSS system, version 16) to estimate mean ± standard error (ST) and ANOVA for the

effects of treatment and time on parameters under investigation (SPSS, 2007).

RESULTS

1.Toxicity test for different concentrations of Montanide Gel 01™ Adjuvant:

All examined concentrations of Montanide Gel 01™ have no toxic effect and causing no deaths in inoculated mice as shown in table (1).

Table 1: Toxicity test of Montanide Gel 01™ in adult mice:

Concentration in media	No. of mice	Dead	Alive
Undiluted	10	0	10
10%	10	0	10
15%	10	0	10
20%	10	0	10
25%	10	0	10

2.Adsorbtion test for selecting the suitable concentration of the adjuvant in tissue culture:

Table (2) showed that the first concentration of Montanide Gel 01™ that showed no cytopathic effect in tissue culture was 20% so we used this concentration in the prepared vaccine and no virus detected after titration.

Table 2: Titre of the virus in the supernatant fluid of the mixture of the different concentration of Montanide Gel 01™ and the Antigen:

The concentration of Montanide Gel 01™	Virus titre (log ₁₀ TCID ₅₀ / 0.1 ml)	CPE after centrifugation	Virus titre in the supernatant (Log ₁₀ TCID ₅₀ / 0.1 ml)
10%	10 ⁷	++	10 ⁵
15%	10 ⁷	+	10 ³
20%	10 ⁷	-	-
25 %	10 ⁷	-	-

3- Quality control of the prepared vaccine:

3.1. Sterility test: revealed that all vaccine preparations are free from any bacterial, fungal or mycoplasmal growth during the period of observation (14 days).

3.2.Potency test in mice:

Both Aluminium hydroxide gel and Montanide Gel 01TM inactivated RVF vaccines gave an acceptable ED₅₀/ML (permissible limit 0.02/ml) as shown in table (3).

Table 3: ED₅₀ / ml for the prepared inactivated RVF vaccines in mice (Permissible limit 0.02/ml):

Type of vaccine	ED ₅₀ \ml
Aluminium hydroxide gel inactivated RVF vaccine	0.0017
Montanide Gel 01TM inactivated RVF vaccine 20%	0.0011

4.Clinical examination of vaccinated sheep:

This including body temperature for 7 days post-vaccination, there were no clinical signs in all vaccinated groups, no post-vaccinal reaction at the site of injection and no elevation of body temperature

5.Evaluation of the humoral immune response in sheep vaccinated with different kinds of inactivated RVF vaccines

5.1. Results of serum neutralization test (SNT):

Table 4: Mean of neutralizing indices of sheep vaccinated with aluminium hydroxide and Montanide Gel 01TM inactivated RVF vaccine:

Animal group	Mean neutralizing indices at different period post vaccination																	Overall mean
	0 day	7 th day	14 th day**	28 th day	2 nd Month	3 rd Month	4 th Month	5 th Month	6 th Month	7 th Month	8 th Month	9 th Month	10 th Month	11 th Month	12 th Month	13 th Month	14 th Month	
Gp (1) vaccinated with Aluminium hydroxide gel Inactivated RVF vaccine (one dose)	0.30 ± 0.01	0.90 ± 0.09	1.74 ± 0.11	2.46 ± 0.15	3.00 ± 0.08	2.88 ± 0.09	2.63 ± 0.07	2.52 ± 0.12	2.16 ± 0.11	1.92 ± 0.07	1.54 ± 0.11	1.32 ± 0.06	0.96 ± 0.06	0.6 ± 0.09	0.6 ± 0.09	0.6 ± 0.15	0.6 ± 0.15	1 ^d ± 0.11
Gp (2) vaccinated with Aluminium hydroxide gel Inactivated RVF vaccine (two doses)	0.39 ± 0.09	0.93 ± 0.15	1.7 ± 0.12	3.32 ± 0.08	3.25 ± 0.09	3.17 ± 0.11	3.0 ± 0.06	2.72 ± 0.12	2.66 ± 0.09	2.50 ± 0.15	2.09 ± 0.09	1.91 ± 0.06	1.75 ± 0.09	1.15 ± 0.15	0.76 ± 0.12	0.6 ± 0.15	0.6 ± 0.09	1.8 ^c ± 0.08
Gp (3) vaccinated with Montanide Gel 01TM inactivated RVF vaccine 20% (one dose)	0.42 ± 0.09	2.04 ± 0.15	2.52 ± 0.06	2.82 ± 0.12	3.52 ± 0.15	3.71 ± 0.09	3.64 ± 0.12	3.54 ± 0.02	3.22 ± 0.15	2.82 ± 0.05	2.52 ± 0.02	2.04 ± 0.09	1.8 ± 0.15	1.70 ± 0.09	1.32 ± 0.12	0.6 ± 0.09	0.6 ± 0.09	2.3 ^b ± 0.11
Gp (4) vaccinated with Montanide Gel 01TM inactivated RVF vaccine 20 % (two doses)	0.32 ± 0.09	2.07 ± 0.12	2.56 ± 0.02	3.01 ± 0.11	3.99 ± 0.15	3.87 ± 0.12	3.70 ± 0.09	3.59 ± 0.08	3.37 ± 0.09	3.19 ± 0.12	2.87 ± 0.11	2.45 ± 0.15	2.29 ± 0.08	2.11 ± 0.12	1.9 ± 0.09	1.7 ± 0.15	1.4 ± 0.09	2.5 ^a ± 0.11
Gp (5) Non-vaccinated control	0.6 ± 0.09	0.6 ± 0.12	0.6 ± 0.08	0.6 ± 0.11	0.75 ± 0.12	0.6 ± 0.15	0.6 ± 0.6	0.9 ± 0.15	0.6 ± 0.15	0.6 ± 0.12	0.6 ± 0.15	0.45 ± 0.09	0.6 ± 0.12	0.6 ± 0.09	0.45 ± 0.15	0.6 ± 0.09	0.72 ± 0.09	0.61 ± 0.02

Values with different alphabetic within the right column showed highly significant difference at P < 0.01.

(**) time of the 2nd dose. Protective level 1.7.

Fig.1: Mean of neutralizing indices of sheep vaccinated with Aluminium hydroxide and Montanide Gel 01™ inactivated RVF vaccine:

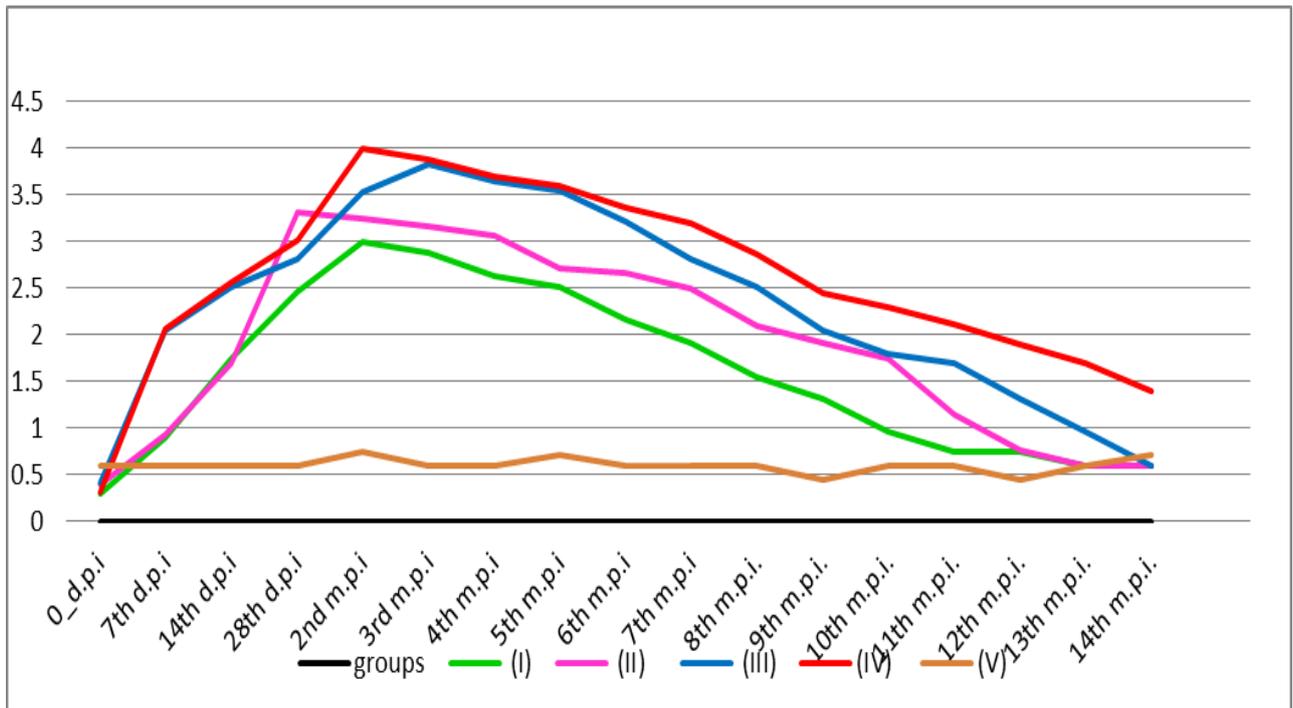
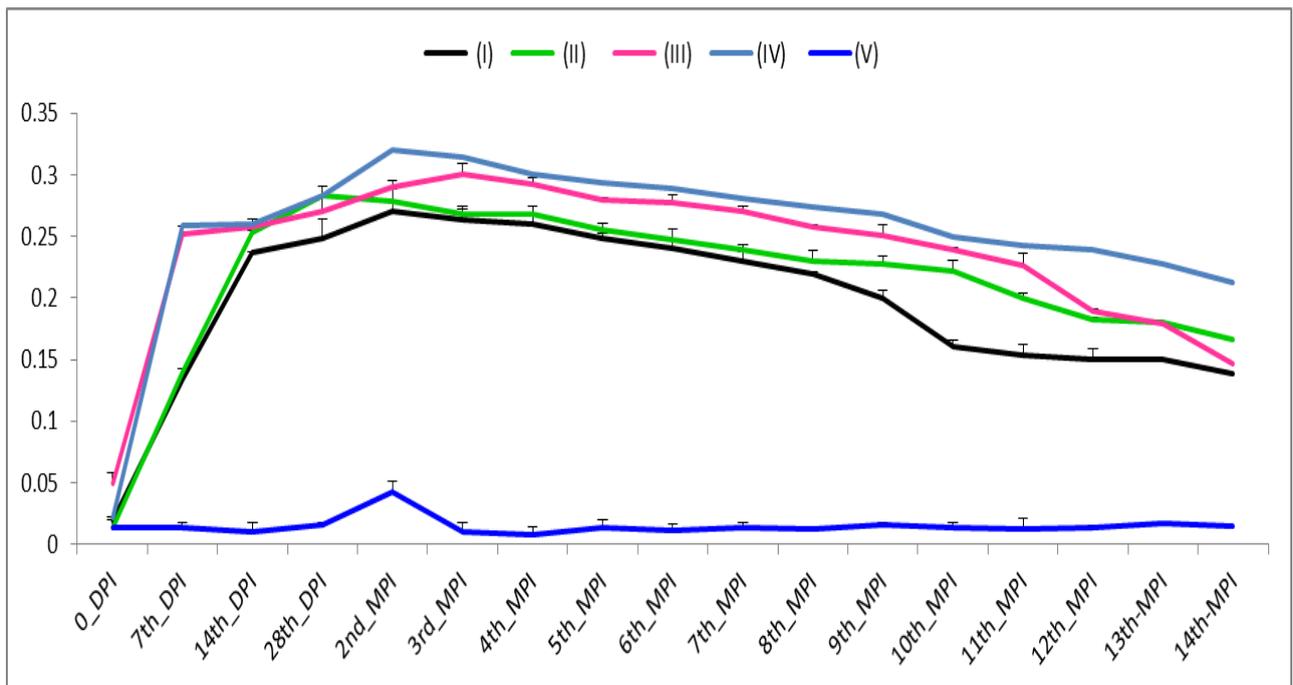


Fig.2: Mean values of ELISA optical density in sheep vaccinated with aluminium hydroxide and Montanide Gel 01™ inactivated RVF vaccine:



5.2. Results of ELISA

Table 5: Mean values of ELISA optical density in sheep vaccinated with aluminium hydroxide and Montanide Gel 01™ inactivated RVF vaccine:

Animal group	Mean optical densities at different period post-vaccination																	
	0 day	7 th day	14 th day (*)	28 th day	2 nd M	3 rd M	4 th M	5 th M.	6 th M.	7 th M	8 th M	9 th M	10 th M.	11 th M.	12 th M.	13 th M	14 th M	Overall mean
Gp (1) vaccinated with Aluminium hydroxide gel Inactivated RVF vaccine (one dose)	0.019 ± 0.001	0.134 ± 0.009	0.237 ± 0.001	0.249 ± 0.015	0.270 ± 0.008	0.263 ± 0.009	0.260 ± 0.007	0.248 ± 0.004	0.240 ± 0.007	0.230 ± 0.007	0.220 ± 0.001	0.200 ± 0.006	0.160 ± 0.006	0.153 ± 0.009	0.150 ± 0.009	0.150 ± 0.006	0.139 ± 0.006	0.188 ^d ± 0.11
Gp (2) vaccinated with Aluminium hy droxide gel Inactivated RVF vaccine (two doses)	0.013 ± 0.009	0.140 ± 0.002	0.253 ± 0.002	0.283 ± 0.008	0.279 ± 0.009	0.268 ± 0.007	0.268 ± 0.006	0.255 ± 0.006	0.247 ± 0.009	0.239 ± 0.004	0.230 ± 0.009	0.228 ± 0.006	0.222 ± 0.009	0.200 ± 0.004	0.183 ± 0.001	0.170 ± 0.009	0.150 ± 0.001	0.192 ^c ± 0.08
Gp (3) vaccinated with Montanide Gel 01™ inactivated RVF vaccine 20% (one dose)	0.049 ± 0.009	0.252 ± 0.006	0.258 ± 0.006	0.271 ± 0.005	0.290 ± 0.005	0.300 ± 0.009	0.292 ± 0.006	0.280 ± 0.002	0.277 ± 0.007	0.270 ± 0.005	0.258 ± 0.002	0.251 ± 0.009	0.239 ± 0.002	0.227 ± 0.009	0.190 ± 0.001	0.179 ± 0.001	0.147 ± 0.009	0.228 ^b ± 0.11
Gp (4) vaccinated with Montanide Gel 01™ inactivated RVF vaccine 20 % (two doses)	0.022 ± 0.009	0.259 ± 0.019	0.260 ± 0.002	0.283 ± 0.001	0.320 ± 0.006	0.314 ± 0.008	0.300 ± 0.009	0.294 ± 0.008	0.289 ± 0.009	0.281 ± 0.009	0.274 ± 0.001	0.268 ± 0.001	0.250 ± 0.001	0.243 ± 0.002	0.239 ± 0.008	0.228 ± 0.009	0.213 ± 0.001	0.281 ^a ± 0.11
Gp (5) Non-vaccinated control	0.013 ± 0.009	0.013 ± 0.004	0.010 ± 0.008	0.016 ± 0.001	0.043 ± 0.008	0.010 ± 0.007	0.008 ± 0.006	0.013 ± 0.007	0.011 ± 0.005	0.013 ± 0.004	0.012 ± 0.002	0.016 ± 0.001	0.013 ± 0.005	0.012 ± 0.009	0.014 ± 0.001	0.017 ± 0.009	0.015 ± 0.001	0.014 ^(e) ± 0.02

6. Evaluation of the cell-mediated immune response in sheep vaccinated with gel inactivated RVF vaccine:

6.1. Lymphocyte blastogenesis:

It was noticed that, cell proliferation expressed by optical density was early high in Gp.3 but more strong in Gp.4 and a slight elevation in Gp.1 and 2 as shown in table (6).

Table 6: Results of lymphocytic blastogenesis assay obtained in different sheep groups after vaccination with RVF prepared vaccines.

Sheep group	No. of sheep	Mean optical densities of proliferation assay						Overall mean
		Days post-vaccination						
		1 st	3 rd	5 th	7 th	10 th	15 th	
Gp (1) vaccinated with Aluminium hydroxide gel Inactivated RVF vaccine (one dose)	5	0.32	0.41	0.47	0.44	0.39	0.34	0.39 ^d
		±	±	±	±	±	±	±
		0.08	0.09	0.11	0.07	0.12	0.09	0.08
Gp (2) vaccinated with Aluminium hydroxide gel Inactivated RVF vaccine (two doses)	5	0.45	0.53	0.64	0.67	0.56	0.47	0.55 ^c
		±	±	±	±	±	±	±
		0.11	0.09	0.09	0.08	0.08	0.09	0.07
Gp (3) vaccinated with Montanide Gel 01TM inactivated RVF vaccine 20% (one dose)	5	0.59	0.66	0.82	0.90	0.88	0.76	0.76 ^b
		±	±	±	±	±	±	±
		0.08	0.07	0.07	0.08	0.09	0.11	0.08
Gp (4) vaccinated with Montanide Gel 01TM inactivated RVF vaccine 20 % (two doses)	5	0.66	0.72	0.90	1.1	1.4	0.83	0.94 ^a
		±	±	±	±	±	±	±
		0.09	0.09	0.08	0.09	0.09	0.11	0.08
Gp (5) Non-vaccinated control	5	0.11	0.09	0.11	0.1	0.12	0.11	0.1 ^e
		±	±	±	±	±	±	±
		0.09	0.08	0.08	0.07	0.09	0.08	0.08

Values with different alphabetic within the right column showed highly significant difference at P < 0.01.

6.2. Phagocytic activity test

Table 7: Results of phagocytic percentage obtained in different sheep groups after vaccination with RVF prepared vaccines.

Sheep group	No. of sheep	Phagocytic percentage (%)						Overall mean
		Days post-vaccination						
		1 st	3 rd	5 th	7 th	10 th	15 th	
Gp (1) vaccinated with Aluminium hydroxide gel Inactivated RVF vaccine (one dose)	5	27	32	36	34	31	28	31.3 ^d
		±	±	±	±	±	±	±
		0.08	0.09	0.11	0.07	0.07	0.11	0.08
Gp (2) vaccinated with Aluminium hydroxide gel Inactivated RVF vaccine (two doses)	5	44	48	54	56	52	48	50.3 ^c
		±	±	±	±	±	±	±
		0.07	0.08	0.09	0.11	0.08	0.07	0.07
Gp (3) vaccinated with Montanide Gel 01TM inactivated RVF vaccine 20% (one dose)	5	62	65	72	78	74	68	69 ^b
		±	±	±	±	±	±	±
		.08	0.07	0.07	0.07	0.08	0.08	0.09
Gp (4) vaccinated with Montanide Gel 01TM inactivated RVF vaccine 20 % (two doses)	5	66	70	74	79	81	83	75 ^(a)
		±	±	±	±	±	±	±
		0.07	0.08	0.11	0.08	0.11	0.09	0.11
Gp (5) Non-vaccinated control	5	21	18	19	20	17	19	19 ^e
		±	±	±	±	±	±	±
		0.07	0.09	0.08	0.09	0.11	0.07	0.07

Values with different alphabetic within the right column showed highly significant difference at P < 0.01.

Phagocytic activity was expressed by phagocytic percentage and phagocytic index in different groups as shown in table (7) and (8). It was noticed that there was early high macrophage activity in Gp.(3) but more strong in Gp.(4) and nearly slight elevation in Gp.(1) and (2).

Table 8: Results of phagocytic indices obtained in different sheep groups after vaccination with RVF prepared vaccines.

Sheep group	No. of sheep	Phagocytic index						Over all mean
		Days post vaccination						
		1 st	3 rd	5 th	7 th	10 th	15 th	
Gp (1) vaccinated with Aluminium hydroxide gel Inactivated RVF vaccine (one dose)	5	0.23 ± 0.08	0.29 ± 0.09	0.36 ± 0.11	0.33 ± 0.07	0.31 ± 0.07	0.24 ± 0.11	0.29 ^d ± 0.08
Gp (2) vaccinated with Aluminium hydroxide gel Inactivated RVF vaccine (two doses)	5	0.31 ± 0.07	0.35 ± 0.08	0.34 ± 0.09	0.37 ± 0.11	0.34 ± 0.08	0.30 ± 0.07	0.33 ^c ± 0.07
Gp (3) vaccinated with Montanide Gel 01TM inactivated RVF vaccine 20% (one dose)	5	0.45 ± 0.08	0.56 ± 0.07	0.66 ± 0.07	0.81 ± 0.07	0.74 ± 0.08	0.65 ± 0.08	0.64 ^b ± 0.09
Gp (4) vaccinated with Montanide Gel 01TM inactivated RVF vaccine 20 % (two doses)	5	0.56 ± 0.07	0.60 ± 0.08	0.74 ± 0.11	0.83 ± 0.08	0.83 ± 0.11	0.85 ± 0.09	0.73 ^a ± 0.11
Gp (5) Non-vaccinated control	5	0.13 ± 0.07	0.11 ± 0.09	0.11 ± 0.08	0.11 ± 0.09	0.12 ± 0.11	0.11 ± 0.07	0.11 ^e ± 0.07

Values with different alphabetic within the right column showed highly significant difference at P < 0.01.

6.3.Detection of interferon γ in the blood of mice using Syber green quantitative real-time PCR

The real-time PCR results revealed that sheep vaccinated with Montanide Gel 01TM inactivated RVF vaccine and boosted 2 weeks apart showed high level of interleukin 6 and 12 as shown in table (9) and (10) and they gradually increased till reach high level in the 7th-day post-vaccination, when compared with the level of interleukin 6 and 12 in groups vaccinated with one dose or two doses of Aluminium hydroxide inactivated RVF vaccine. The control results revealed no interleukin 6 and 12 gene expression

Table 9: Detection of interlukin12in blood of sheep using Syber green quantitative real-time PCR:

Sheep group	No. of sheep	Cycle threshold of Interleukin 12 gene expression						Overall mean
		Days post-vaccination						
		1 st	3 rd	5 th	7 th	10 th	15 th	
Gp (1) vaccinated with Aluminium hydroxide gel Inactivated RVF vaccine (one dose)	5	36.430 ± 0.657	34.932 ± 0.254	32.010 ± 0.521	34.000 ± 0.254	35.910 ± 0.162	36.250 ± 0.236	34.922 ^a ± 0.524
Gp (2) vaccinated with Aluminium hydroxide gel Inactivated RVF vaccine (two doses)	5	35.192 ± 0.296	33.817 ± 0.056	31.910 ± 0.361	31.300 ± 0.251	32.714 ± 0.435	33.913 ± 0.254	33.141 ^b ± 0.566
Gp (3) vaccinated with Montanide Gel 01TM inactivated RVF vaccine 20% (one dose)	5	35.000 ± 0.145	32.110 ± 0.523	29.988 ± 0.357	30.179 ± 0.562	31.876 ± 0.325	32.333 ± 0.524	31.194 ^c ± 0.516
Gp (4) vaccinated with Montanide Gel 01TM inactivated RVF vaccine 20 % (two doses)	5	34.349 ± 0.235	31.919 ± 0.285	28.791 ± 0.342	28.001 ± 0.268	29.899 ± 0.666	31.189 ± 0.522	30.691 ^d ± 0.666
Gp (5) Non-vaccinated control	5	No Cycle threshold						

Primer dimer (Real-time negative control (mix+H2o+primers): 36.32.

Values with different alphabetic within the right column showed highly significant difference at P < 0.01.

Table 10: Detection of interleukin 6 in the blood of sheep using Syber green quantitative real-time PCR:

Sheep group	No. of Sheep	Cycle threshold of Interleukin 6 gene expression						Overall mean
		Days post-vaccination						
		1 st	3 rd	5 th	7 th	10 th	15 th	
Gp (1) vaccinated with AIOH gel Inactivated RVF vaccine (one dose)	5	37.111 ± 0.647	36.399 ± 0.666	34.197 ± 0.388	35.911 ± 0.196	36.768 ± 0.250	37.318 ± 0.813	36.284a ± 0.625
Gp (2) vaccinated with AIOH gel Inactivated RVF vaccine (two doses)	5	36.401 ± 0.574	35.760 ± 0.035	33.312 ± 0.250	32.719 ± 0.388	34.714 ± 0.289	33.000 ± 0.745	34.317b ± 0.564
Gp (3) vaccinated with Montanide Gel 01 TM inactivated RVF vaccine 20% (one dose)	5	36.011 ± 0.546	34.310 ± 0.357	31.990 ± 0.284	31.011 ± 0.384	33.100 ± 0.458	32.985 ± 0.168	33.234c ± 0.359
Gp (4) vaccinated with Montanide Gel 01 TM inactivated RVF vaccine 20 % (two doses)	5	35.313 ± 0.512	33.186 ± 0.215	31.043 ± 0.251	30.703 ± 0.382	31.723 ± 0.142	32.001 ± 0.325	32.328d ± 0.384
Gp (5) Non-vaccinated control	5	No cycle threshold						

Primer dimer (Real-time negative control (mix+H₂O+primers): 35.482

Values with different alphabetic within the right column showed highly Significant difference at P < 0.01.

DISCUSSION

The progress in vaccine production is directed towards the selection of the proper adjuvant that can elaborate high and long-standing immunity. Adjuvants are considered one of the important factors in vaccine formulations as it can influence the immunity and increase the immune response referred to the vaccine. So this study was applied to evaluate the immune response and duration of immunity in sheep vaccinated with 20% Montanide Gel 01TM inactivated RVF vaccine in comparison to aluminium hydroxide gel inactivated RVF vaccine Toxicity test of Montanide Gel 01TM adjuvant were done by I/P inoculation of adult mice, with four concentrations of Montanide Gel 01TM (10%, 15%, 20 and 25%) which revealed no signs of toxicity during 10 days post-inoculation as shown in table (1).

The results of adsorption test revealed that the most suitable concentration of Montanide Gel 01TM was 20% as it was the first concentration showed no cytopathic effect of the supernatant after centrifugation when inoculated in tissue culture and no virus detected by titration as shown in table (2). Sterility test showed that the vaccines were free from any bacterial, fungal and mycoplasma contaminations, these results agreed with **OIE (2012)** that revealed the vaccine batch must be free from bacteria, fungus and mycoplasma. by applying clinical examination on the vaccinated sheep, no abnormal signs were observed, no elevation of body temperature and no postvaccinal reaction at the site of injection.

Evaluating the potency of the prepared vaccines in adult mice as shown in table (3), revealed that the vaccines gave protection in mice with ED₅₀/ml (0.0017 and 0.0011/ml) for the inactivated RVF vaccines with aluminium hydroxide gel, Montanide Gel 01TM 20%) respectively. Serum neutralization test revealed that sheep vaccinated with one dose Aluminum hydroxide inactivated RVF vaccine showed that the neutralizing antibodies started to rise from the 1st-week post-vaccination (0.9 log₁₀) and reached the protective level (1.7 log₁₀) on the 2nd week post-vaccination as shown in table (4), and then reached the peak (3 log₁₀) at the 2nd month post-vaccination and continue in protective level till 8th month post-vaccination, these results were in agreement with those obtained by **El Nimr (1980)** and **Eman (1995)** who recorded that the protective NI level obtained by the inactivated vaccines within 2 weeks post-vaccination, the neutralizing antibody reaches the peak (2.6 logs₁₀) at the 2nd month post-vaccination and continue within the protective level till 6th month post-vaccination.

This results agreed with that obtained by **Heba, (2013)** who found protective antibody level of sheep vaccinated with inactivated RVF vaccine till 7 months post-vaccination while in sheep vaccinated with two doses of aluminium hydroxide gel, the NI level reached the protective level (1.7 log₁₀) at the 2nd-week post-vaccination and reached to the peak at 2nd-month post-vaccination and continue in protective level till the 10th-month post-vaccination.

In sheep vaccinated with one dose and two doses of Montanide Gel 01TM inactivated RVF vaccines (20%), the neutralizing antibodies started to rise after vaccination to reach its protective level (2.04 log₁₀ and 2.07 log₁₀) respectively on the 1st week post-vaccination, and increased gradually to its peak (3.82 log₁₀) at the 3rd month post-vaccination in sheep vaccinated with one dose and in sheep vaccinated with two doses reached the peak at 2nd month post-vaccination (3.99 log₁₀) and stay within the protective level (1.7 log₁₀ and 1.7 log₁₀) respectively at the (11th month and 13th-month post-vaccination respectively as shown in table (4).

These results in concordance with those reported by **Dupuis et al., (2008)** who observed that using of Montanide Gel 01TM as adjuvant gave sufficient early immune response in vaccinated cattle and with **El Sayed et al., (2011)** who found that Montanide Gel 01TM adjuvant vaccine gives an intense immune response and showed prolongation of antibody secretion in comparison to the aluminium hydroxide. Also, our results agreed with **Deville et al., (2008)**; **Parker et al., (2009)**; **Deville et al., (2011)** and **Abul-Magd (2014)** who reported that antibodies production induced by Montanide Gel 01TM based vaccine was higher than aluminium based vaccines.

Moreover, confirmation of humoral immune response was assessed by ELISA as shown in table (5) as it is considered the sensitive and accurate test to evaluate the immune status of RVF in animals sera (**Taha et al., 2002** and **Fafetine et al., 2007**). When the ELISA test was done on the same serum samples, the results were in parallel course with that obtained by SNT. Similar findings were reported by **Eman, (1995)** and **Mona, (2008)**.

Results of cell proliferation expressed by optical density were high in sheep vaccinated with one dose of Montanide Gel 01TM inactivated RVF vaccine but more strong in sheep vaccinated with two doses of Montanide Gel 01TM inactivated RVF vaccine as shown in table (6) . While in sheep vaccinated with one dose of AL(OH)₃ gel inactivated RVF vaccine showed slight elevation of cell proliferation and these results agreed with that obtained by **Hammad (2015)** who recorded slight elevation in sheep vaccinated by RVF vaccine with Aluminium hydroxide gel.

Results of phagocytic activity expressed by phagocytic percentage and phagocytic index showed early significant in sheep vaccinated with one dose of Montanide Gel 01TM inactivated RVF vaccine as shown in table (8) and (9) but more strong in sheep vaccinated with two doses of Montanide Gel 01TM inactivated RVF vaccine and nearly no significance in sheep

vaccinated with one dose of AL (OH)₃ gel inactivated RVF vaccine which agreed with **Hammad, (2015)**.

IL 6 and IL 12 play an important role in the generation of cell-mediated immunity (**Irene et al., 1999**), So in this study, by using RT-PCR the level of IL6 and IL12 gene expression was detected in sheep vaccinated with different doses of Montanide Gel 01TM inactivated RVF vaccine. The results revealed that two doses of Montanide Gel 01TM inactivated RVF vaccine induced a higher level of IL 6 and IL12 gene expressions than Aluminium hydroxide gel inactivated RVF vaccine as shown in table (10).

CONCLUSION

From the obtained results, it can be concluded that, Montanide Gel 01TM was highly immunogenic when used as adjuvant in preparation of RVF vaccine inducing a higher level of antibody titre with prolonged duration of immunity, higher cell proliferation and phagocytic activity and IL6 and IL12 level so allowing a better control of the disease, so we recommend it to be used in animal vaccination to increase the level and duration of immune response.

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