Immunogenicity and Keeping Quality Studies of Inactivated Rift Valley Fever Oil Vaccines

Diana M.A. Ezzaldeen, Atwa M. H*, Mona A. El-Manzalawy, Taradi A. S1, Hassan K.E.Z, Marwa Y. H

Veterinary Serum and Vaccine Research Institute, Abbasia, Cairo, Egypt

Abstract: This work aims to improve and enhance the immunogenicity of the locally produced inactivated RVF vaccine to induce high level of elongated duration of immunity in vaccinated animals using Montanide oils adjuvant (ISA 206 and ISA 50). Validity and keeping quality for all forms of the prepared vaccines were evaluated by measuring the ED$_{50}$/ml at different intervals of preservation at 4°C. Twenty susceptible sheep were used in this study divided into four groups(five sheep/group) vaccinated with Aluminum Hydroxide gel inactivated RVF vaccine (group-1), Montanide Oil ISA 206 inactivated RVF vaccine (group-2), Montanide Oil ISA 50 inactivated RVF vaccine (group-3) and unvaccinated control group (group-4). The humoral immunity in vaccinated sheep was evaluated using serum neutralization test (SNT) and enzyme linked immunosorbant assay (ELISA) where the protective immune response was detected in group (2) by serum neutralization test at 2nd week post vaccination while in group (3) it was detected at the 1st month post vaccination with values of 1.8 log 10 and 2.8 log 10 respectively and stayed within protective till the end of 10 months in Group 2 and 14th months in Group 3 with values of 1.7 log 10 and 1.7 log 10 respectively. These results was correlated to that obtained by ELISA indicating that Montanide Oil ISA 50 is the preferable oil adjuvant to the inactivated RVF vaccine inducing the highest and longest protective immune levels in vaccinated animals.

Keywords: RVF, SNT, ELISA, MONTANIDE OIL, ISA 206, ISA 50

1. INTRODUCTION

RVF is a viral zoonosis that primarily affects domestic animals (including cattle, sheep, camels and goats) and to a lesser extent human. The RVF virus is highly contagious for humans when handling potentially-infected livestock. Raising awareness of the risk factors of RVF transmission as well as protective measures such as vector control and protection against mosquito bites in addition to vaccination, are the key to reduce human infection and deaths (WHO, 2019).

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 humeral and cell mediated immunity (Dalsgard et al., 1990).

RVFV circulation in most African countries and certain Middle Eastern countries has been documented by serological surveys, animal and human cases and outbreak reports (Clements et al., 2016 and Redding et al., 2017). Since 2000, epizootic events associated with human cases have been reported in Saudi Arabia and Yemen (2000–2001), Egypt (2003), Kenya, Tanzania, Somalia and Sudan (2006–2007); Madagascar (2008–2009), South Africa and Namibia (2009–2011); Mauritania (2010, 2012–2015); Senegal (2013–2014); Uganda and Niger (2016); Kenya (2018), South Sudan (2018) (WHO 2018, Nyakarahuka et al., 2019 and WHO 2019) and in South Africa; (OIE 2018) reported human RVF cases associated with an isolated outbreak on a sheep farm in the Free State Province of South Africa at the end of the 2017–2018 in the mosquito season and last recorded RVF cases were in Sudan 2019 with 47 infected cases including 2 deaths (WHO 2019).

Vaccine adjuvant is a very important factor which stimulates specific components of either humeral or cell-mediated immune response (Lombard et al., 2007). Selecting the ideal or the most suitable adjuvant is one of the
important tools in improving the efficacy of the RFV vaccine. An ideal adjuvant is one which can stimulate the humoral immune response early, and promote production of high antibody titers that would last long. It should also stimulate the cellular immune response (Daoud et al., 2013 and Ibrahim 2011). The oil adjuvant has the capability for generating a rapid, high and long-lasting immune response. Generally, the Montanide series of oil adjuvants (SEPPIC, France) has a clear immunological effect for inactivated vaccine in different susceptible animals (Dara et al., 2013 and Patil et al., 2002).

The present study evaluates in a comparison the efficacy of different oil based adjuvants used in RVF vaccine formulation in addition to Aluminum Hydroxide gel to select the best adjuvant which could be able to induce high long duration of immunity in vaccinated sheep.

2. MATERIALS AND METHODS

2.1. Experimental Animals

2.1.1. Suckling Mice

Ten of three to five days old baby Swiss Albino mice were used test the safety of the prepared inactivated RVF vaccines.

2.1.2. Weand mice

Swiss Albino weaned mice of 21-30 days old were used for evaluating the validity of the prepared vaccines. All mice were supplied by the Lab Animal Breeding Unit, Veterinary Serum and Vaccine Research Institute (VSVRI), Abbasia, Cairo, Egypt.

2.1.3. Sheep

Twenty local breed sheep of 3-4 months old were used for evaluation of their immune response to the prepared vaccines. All of these animals were screened using serum neutralization test (SNT) 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 apparently healthy and free from external and internal parasites.

2.2. Tissue Culture Cells

Baby Hamster Kidney cells (BHK21) were obtained from VSVRI, Abbasia, and maintained in Eagles minimum essential medium with 8-10% bovine serum as described by Xuan et al., (2011), and were used for virus propagation, vaccine production and SNT.

2.3. Virus

The original Rift Valley Fever (RVF) virus was that isolated from a human patient in Zagazig, Sharkia Province and supplied by NUMRU -3. It was designated as ZH501 with a titer of $10^{7.5}$ TCID$_{50}$ / ml and used in vaccine preparations and serological tests.

2.4. Samples

Serum samples were collected from sheep before vaccination and after vaccination until the end of the experiment. Sera were stored at –20°C and inactivated at 56°C for 30 minutes before being used in the serological studies (SNT and ELISA).

2.5. Adjuvants

2.5.1. Aluminium Hydroxide Gel

It was obtained from (Alliance Bio Company, USA), Lot. No. 11-274-30 and used in concentration of 20% as adjuvant for RVF inactivated vaccine preparation.

2.5.2. Montanide ISA Oils

Montanide oils ISA 206 and ISA 50 were supplied by Seppic, Paris, France) resembling a group of oil/ surfactant based adjuvants were used for preparation of experimental inactivated RVF vaccines.

2.6. Vaccine Preparation

The used procedure of vaccine preparation included the following steps:

2.6.1. Propagation of the Vaccinal Virus

Confluent BHK cell sheets in Roux flasks were inoculated with the RVF virus and incubated at 37°C and examined daily for cytopathic effect (CPE) which usually occurred after 48-72 hours. The flasks were subjected twice to freezing and thawing and then the harvest was aseptically mixed together and centrifuged for 10 minutes at 3000 rpm in a cooling centrifuge. The supernatant fluids constituted the required virus for vaccine preparation. This virus was tested for its sterility as well as titrated in tissue culture and in 3-4 weeks old adult mice.

2.6.2. Virus Inactivation

RVF virus was inactivated with Binary (2-Bromoethyle ammonium bromide with sodium hydroxide) according to Blackburn and Besselaar (1991) as follow:

RVF virus grown in BHK monolayer culture having a titer of $10^{7.5}$ TCID$_{50}$/ ml in tissue
culture was mixed with 1% of 0.1 M BEI in 0.2 N NaOH solution to give a final concentration of 0.001 M of BEI. The virus and BEI mixture was mixed well and placed in a water bath at 37°C till complete inactivation then 2% sodium thiosulphate solution was added to neutralize the residual BEI and stopping its over action.

2.6.3. Safety Test in Mice

The safety test of the prepared inactivated virus was performed by intracerebral (I/C) inoculation of 10 suckling mice (3 – 5 days old) each with 0.03 ml of inactivated virus and subjected to daily observation for 10 days for any signs of RVF disease or deaths according to El-Nimr (1980) and Eman (1995).

2.6.4. Vaccine Formulation

Three formula of inactivated RVF vaccine were prepared as follow:

Formula-1: using 20% Aluminium hydroxide gel 20% as adjuvant

Formula-2: using Montanide oil ISA 206 as adjuvant

Formula-3: using Montaniode oil ISA 50 as adjuvant

2.6.5. Evaluation of the Prepared Vaccine Formulae

2.6.5.1. Sterility Test

The freedom of the prepared inactivated RVF vaccines from foreign contaminants (Mycoplasma, aerobic, and anaerobic bacteria, and fungi) was tested at the Rift Valley Fever Department, VSVRI, Abbasia, Cairo.

2.6.5.2. Potency Test

Fivefold dilutions of each vaccine formula were prepared in suitable media starting from 1:1 to 1:625. Five groups of weaned mice (21-28 days old) were used for each dilution and each mouse was inoculated with two doses 0.2 ml of inactivated virus I/P, one week a part. Seven days after the second inoculation, all animals were challenged via intraperitoneal (I/P) route with 0.1 ml of RVF virus containing $10^6$ MIPLD50 / ml this was in addition to other two groups of mice, one group inoculated with challenge virus as positive control and the other group was kept as non vaccinated non challenged negative control. All groups of mice were kept under observation for 21 days and deaths were recorded daily. The ED$_{50}$ / ml was calculated according to the method of Reed and Muench (1938). Deaths occurred during the first day were considered non specific.

2.6.6. Determination of the Validity of the Oil Vaccines

Vaccine validity was determined by estimating the ED$_{50}$ of the two oil vaccines at 4° and 25° C (room temperature) by applying potency test at different periods in comparison with the Aluminium hydroxide inactivated RVF vaccine (The permissible limit of ED$_{50}$ is 0.02 as reported by Randall et al., 1962 and Gihan et al., 1998).

2.7. Scheme of Sheep Vaccination

Each sheep group included 5 animals managed as follow:

Group-1: Each sheep was inoculated S/C with 1ml of inactivated aluminum hydroxide gel adjuvanted RVF vaccine.

Group-2: Inoculated S/C with 1ml/animal of ISA 206 oil adjuvanted inactivated RVF vaccine.

Group-3: Inoculated S/C with 1ml/animal of ISA 50 oil adjuvanted inactivated RVF vaccine.

Group-4: was kept as non vaccinated (control negative).

2.8. Sampling

Serum samples were obtained from all sheep before and after vaccination at intervals of 7, 14, 21 days and then monthly for 15 months post vaccination and kept at –20° C until used for serological assays.

2.9. Evaluation of the Humoral Immune Response

Collected serum samples from vaccinated and non vaccinated sheep were tested for the presence of antibody against RVF virus by SNT using the technique calculated the antibody titer as described by Reed and Muench (1938) and indirect ELISA according to Voller (1976).

2.10. Statistical Analysis

The obtained data were statistically analyzed by (SPSS system, version16) to estimate mean ± standard error (SE) and ANOVA for the effects of treatment and time on parameters under investigation SPSS 2007: statistical analysis: version 16.

3. RESULTS AND DISCUSSION

This study was carried out to evaluate the immunogenicity and duration of immunity in sheep vaccinated with different preparations inactivated RVF vaccine with different
montanide oil adjuvants in comparison with the aluminum hydroxide gel inactivated vaccine.

Through the present work it was found that all prepared inactivated RVF vaccine formulae were free from foreign contaminants (aerobic and anaerobic bacteria; fungi and mycoplasma) as tested by the Rift Valley Fever Department and safe inducing no mortalities or any signs of illness (no allergic reaction, no inflammation, no granuloma, no swelling, no sterile abscess or fever) were observed on inoculated suckling mice during 10 days of observation period.

Regarding the use of oils as adjuvants to the inactivated RVF vaccine; viscosity of such oils is considered as an important factor during selection of a suitable adjuvant, as the high viscosity can results in undesirable side effect as postvaccinal reaction. Table (1) demonstrates the viscosity of the use Montanide oil adjuvants used in this study as stated by manufacture (Seppic, Paris) at 25°C as 30mPa.s for the montanide ISA206 and 200 mPa.s for Montanide ISA 50 as it was concluded that the use of oils as vaccine adjuvants should be of low viscosity as stated Bomford (1997).

### Table 1. Montanide oil viscosity

| Vaccine Component                  | Vaccine 1 | Vaccine 2 |
|-----------------------------------|-----------|-----------|
| Antigen                            | Rift valley fever virus ZH501 |
| Adjuvant (montanide)               | ISA 206   | ISA 50    |
| Type of emulsion                   | Water-in-oil-in-water       | Water-in-oil |
| Oil viscosity at 25°C              | 30        | 200       |

Table (2) showed that all prepared inactivated RVF vaccines had an acceptable ED50/ML (Permissible limit: not more than 0.02/ml) in agreement with what reported by Randall et al (1962) and Gihan et al (1998).

### Table 2. ED50/ML of the prepared inactivated RVF vaccines in mice

| Type of vaccine                                      | ED50/ML   |
|------------------------------------------------------|-----------|
| Aluminium hydroxide gel inactivated RVF vaccine       | 0.0017    |
| Montanide oil (ISA 206) inactivated RVF vaccine       | 0.0013    |
| Montanide oil (ISA 50) inactivated RVF vaccine        | 0.0010    |

Clinical examination of vaccinated sheep including body temperature for 7 days post vaccination, showed no clinical signs except 2 sheep in the group vaccinated with montanide oil ISA 50 which showed post vaccinal reaction at the site of injection with no elevation of body temperature as shown in table (3) confirmed the safety of the prepared vaccines in agreement with the recommendations of Marwa (2015).

### Table 3. Body temperature of vaccinated sheep in different groups

| Animal Group                                      | Mean Temperature n Degree |
|---------------------------------------------------|---------------------------|
|                                                   | Before Vaccination        | Days Post Vaccination |
|                                                   |                          | 1st | 2nd | 3rd | 4th | 5th | 6th | 7th |
| G(1) vaccinated with Aluminium hydroxide gel inactivated RVF vaccine | 39.4 | 39.5 | 39.4 | 39 | 39.7 | 39.4 | 39.4 | 38.9 |
| G(2) vaccinated with Montanide oil ISA 201 inactivated RVF vaccine | 39.5 | 39.3 | 39.5 | 38.9 | 39.1 | 39.4 | 39.4 | 38.9 |
| G(3) vaccinated with Montanide oil ISA 50 inactivated RVF vaccine | 39.4 | 39.2 | 38.8 | 38.9 | 39 | 39.5 | 39.6 | 39.4 |
| G(4) control non vaccinated                        | 39.4 | 39.6 | 39.7 | 39.3 | 39.5 | 39.6 | 39.5 | 39.6 |

Evaluation of the humoral immune response in sheep vaccinated with different preparations of inactivated RVF vaccine through application of SNT and ELIZA on their serum samples revealed the tabulated results in tables (4&5) and fig (1&2). It was noticed that the results obtained by ELIZA came in parallel to that obtained by SNT. The SNT and ELIZA antibody titer against RVFV were negative along the whole experiment in the non vaccinated control sheep. Serum neutralization index in vaccinated sheep with Montanide ISA 206 began to increase from 0.18 before vaccination to reach its protective value (1.8) by the 14th day post vaccination to record its peak (4.8) by the 4th month then began to decrease to be unprotected (1.3) by the 10th month post vaccination. Sheep vaccinated with Montanide ISA50 vaccine showed NI of 0.31 before vaccination recorded a protective level (2.8) at the 1st month with a peak level (5.8) at the 4th month post vaccination then began to decrease to non protective level (1.43) by the end of the 14th month.

From tables (4) and (5) concerning the duration of the protective immunity for RVF virus, there
was a significant difference at \( P < 0.01 \) among the different types of used oil adjuvants. The recorded higher levels of RVF antibodies in vaccinated sheep with oil adjuvants than in those vaccinated with aluminum hydroxide gel vaccine could be attributed to the fact that oil adjuvants have the capability of generating a rapid, high and long lasting immune response and the montanide oil adjuvants (SEPPIC, France) have a clear immunological effect for inactivated vaccines in different susceptible animals (Dara et al., 2013 and Patil et al., 2002).

The efficacy of Montanide adjuvant is not based on one mechanism but really on different modes of action. As emulsion have depot effect and induce a slow release of the antigen, they can recruit immune competent cells by micro diffusion of the droplets via the lymphatic system, or facilitate the antigen uptake by the antigen presenting cells, or at least playing the role of antigen presenting cells. Other mechanism such as lymphocyte trapping and modification of the cell membranes, emulsion is the result of oil phase, established with surfactant and able to modify the cell membranes or to draw together the antigen and antigen presenting cells (Aucotuier and Ganne, 2002).

These findings agree with those of Fakhry et al (2012) and Ehab et al (2015) who found that the duration of FMD immunity induced by Montanide oils ISA 206 and 50 reached 32 and 38 weeks post vaccination respectively. Also our results agree with El-Bagoury et al (2013) who reported that the duration of RVF immunity induced by Montainide oil ISA 206 reached 44 weeks post vaccination.

Table 4. Mean RVF neutralizing indices of vaccinated sheep

| Animal group | 0 day | 9th day | 14th day | 21st day | 1st M | 2nd M | 3rd M | 4th M | 5th M | 6th M | 7th M | 8th M | 9th M | 10th M | 11th M | 12th M | 13th M | 14th M | 15th M | Overall mean |
|--------------|-------|--------|---------|---------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------------|
| Grp (1) vaccinated with Aluminium hydroxide gel | 0.01 | 0.09 | 1.74 | 2.1 | 2.46 | 3.22 | 3.02 | 2.82 | 2.82 | 2.16 | 1.92 | 1.64 | 1.36 | 0.96 | 0.66 | 0.60 | 0.5 | 0.48 | 1.25 (c) 0.11 |
| Grp (2) vaccinated with Inactivated RVF vaccine | 0.18 | 1.60 | 1.8 | 2.0 | 3.6 | 4.3 | 4.8 | 4.3 | 3.8 | 3.2 | 2.9 | 2.2 | 1.7 | 1.3 | 1.3 | 1.2 | 1.2 | 1.2 | 0.75 | 2.2 (b) 0.08 |
| Grp (3) vaccinated with Montanide oil ISA 206 | 0.09 | 0.16 | 0.12 | 0.11 | 0.08 | 0.09 | 0.11 | 0.06 | 0.12 | 0.09 | 0.15 | 0.09 | 0.06 | 0.09 | 0.16 | 0.09 | 0.12 | 0.12 | 0.12 | 0.31 (a) 0.16 |
| Grp (4) vaccinated with Montanide oil ISA 40 | 0.09 | 0.16 | 0.06 | 0.11 | 0.12 | 0.15 | 0.09 | 0.12 | 0.02 | 0.12 | 0.09 | 0.09 | 0.16 | 0.12 | 0.15 | 0.15 | 0.15 | 0.15 | 0.15 | 0.15 (a) 0.16 |
| Grp (5) vaccinated with Montanide oil ISA 200 | 0.09 | 0.12 | 0.09 | 0.11 | 0.11 | 0.12 | 0.09 | 0.12 | 0.09 | 0.12 | 0.09 | 0.09 | 0.15 | 0.12 | 0.12 | 0.12 | 0.12 | 0.12 | 0.12 | 0.68 (a) 0.03 |

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

Fig 1. Mean RVF neutralizing indices of sheep vaccinated with aluminum hydroxide and montanideoils inactivated RVF vaccines
Table 5. Mean values of ELISA optical density in sheep vaccinated with Aluminum hydroxide and Montanide oils inactivated RVF vaccines

![Image of Table 5]

- Values with different alphabetic within the right column showed highly significant difference at $P < 0.01$.
- Cut off: 0.230

Fig2. Mean values of ELISA optical density in sheep vaccinated with Aluminium hydroxide and Montanide oils inactivated RVF vaccines

On the other side evaluation of validity of the prepared inactivated RVF vaccine formulae kept at 4° and 25°C; table (6) showed that by estimating the effective dose fifty (ED50/ml), all forms of RVF oil vaccines were valid within the permissible limit (0.02ED50/ML) up to 12 months except aluminium hydroxide gel which still valid up to 9-10 months only in agreement with the results recorded by Randall et al., (1962) and Gihan et al., (1998). Table (7) showed that the ISA 206 oil vaccine kept its potency at 25° C for at least 4 weeks while the ISA 50 oil vaccine stayed within the permissible limit till the 5th week.

So it could be concluded that 4°C is the best temperature for storage of the oil inactivated RVF vaccines as they were valid for one year, but under recommended temperature 25 °C they stayed valid for weeks in agreement with Samira et al. (1999) and Ehab et al.(2015).

Table6. Validity of different preparations of RVF vaccine at 4 °C

| Type of Vaccine          | Time Post Vaccination |
|--------------------------|-----------------------|
|                          | Zero day | 2nd m  | 4th m  | 6th m  | 7th m  | 8th m  | 9th m  | 10th m | 11th m | 12th m |
| Aluminium hydroxide      | 0.0017   | 0.0017 | 0.0040 | 0.0068 | 0.0097 | 0.0147 | 0.0203 | 0.0248 | 0.0300 | 0.0320 |
| Inactivated RVF vaccine  |          |        |        |        |        |        |        |        |        |        |
| Montanide ISA 206 oil    | 0.0012   | 0.0012 | 0.0011 | 0.0010 | 0.0009 | 0.0007 | 0.0008 | 0.0006 | 0.0007 | 0.0009 |
4. CONCLUSION
All the present obtained results gave the priority to the prepared inactivated montanide oil ISA 206 and ISA 50 RVF inactivated vaccines over the local inactivated aluminum hydroxide gel adjuvanted RVF vaccine.

Montanide oils (206 and 50) RVF vaccines induce long lasting immunity ranged between 10 and 14 months post vaccination. Further, studies are recommended to evaluate the effect of these Montanide oils on the cellular immune response

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