EFFECACY OF MONTANIDE GEL INACTIVATED RVF VACCINE IN COMPARISON WITH ALUMINUM HYDROXIDE GEL INACTIVATED ONE

Diana M. Abul Magd[1], Mohamed Ebeid[2], Abdel-Moneim M. Moustafa[2], Eman Mohamed Sayed Shalakamy[2], Mohamed G. Abdel Wahab[3]

[1]*Rift Valley Fever Unit, Veterinary serum and Vaccine Research Institute, Abbassia, Cairo , Egypt.*
[2]*Infectious Diseases, Department of Animal medicine, Faculty of Veterinary Medicine, Benha University, Egypt*

ABSTRACT

In this work, 20% concentration of montanide gel adjuvanted inactivated RVF vaccine were prepared, and aluminum hydroxide gel RVF vaccine. The prepared vaccines were safe when inoculated into mice with no deaths or onset of symptoms. The potency of the prepared vaccines in adult mice, revealed that the vaccines gave a protection in mice with ED_{50}/ML (0.0017, and 0.0011 ED_{50}/ML) for the inactivated RVF vaccines with aluminum hydroxide gel, and montanide gel 20%) respectively. Both forms of inactivated RVF vaccine tested for its shelf life during storage 12 months at 4˚c, montanide gel 01 RVF vaccines were within the permissible limit (0.02 ED_{50}/ ml). Results of lymphocytic count cleared that montanide gel inactivated RVF vaccine gave early and higher value of lymphocytic count than Aluminum hydroxide gel inactivated RVF vaccine. The results of estimated interferon γ gene expression revealed that montanide gel induced higher level of interferon γ gene expression than Aluminum hydroxide gel. The previous data clearly showed that montanide gel was highly immunogenic than Aluminum hydroxide gel inactivated one.

Key words: ED_{50}: Effective Dose 50

(BVMJ-27(1):239-247, 2014)

1. Introduction

Rift Valley Fever (RVF) is an acute febrile arthropod-borne viral disease. It is a zoonotic disease, highly infectious, and highly fatal among livestock. It is responsible for great loss due to abortions and heavy mortalities in young animals (Easterday et al., 1962 and Digoutte and Peters 1989). RVF disease is caused by Rift Vlley Fever virus of the genus Phlebovirus and transmitted by mosquitoes (OIE 1989). Control of RVF disease in Egypt depends mainly on vector control and vaccination, (Abdel Gaffar et al., 1979). This study was planned to compare the immune response of montanide gel inactivated RVF vaccine 20% with locally produced killed vaccine.

2. Material and Methods

2.1. Materials:

2.1.1. Virus:

Rift Valley Fever (RVF) virus isolated from a human patient in Zagazig, Sharkia province and supplied by NUMRU -3 after being identified to be RVF virus, it was twicely passaged intracerebrally into suckling mice. It was designated as ZH_{501} and had a titer of $10^{7.5}$ $TCID_{50}$ / ml; it was used for preparation of RVF vaccines and in challenge test, it was kindly supplied by RVF department Ser. & Vacc. Res. Inst. Abbasia, Cairo.

2.1.2. Tissue Culture Cells:
Baby Hamster Kidney (BHK_{21}) cells. Were grown and maintained according to Macpherson and Stocker (1962). It was used for application of RVF virus titration, and vaccine preparation.

2.1.3. Unweaned baby mice: Three-four days old mice were used for safety test of the vaccines.

2.1.4. Adult mice: Swiss albino weaned mice, 21-28 days old, were used for titration of the virus, testing the potency of the prepared vaccine and challenge test.

2.1.5. Adjuvants:
A. Aluminum hydroxide gel: The gel was obtained from (Alliance Bio Company, USA), Lot. No. 11-274-30 and was used in concentration 20% for preparation of inactivated RVF vaccine.
B. Montanide gel: It used for preparation of montanide gel inactivated RVF vaccine and It was obtained from Seppic, Paris, France.

2.1.6. Fycol hypaque: (Lymphocyte separation medium) This medium was used for separation of mononuclear leukocyte cells from peripheral blood.

2.1.7. Reagents for total RNA extraction from mice lymphocytes: Trizol reagent (Invitrogen, Carlsbad, CA), Phenol crystals, Trisbase, Distilled water, Concentrated HCL to adjust PH of Tris HCL, Chloroform, Isopropyl alcohol, 75% ethanol in RNA free water and RNA free water.

2.1.8. Reagents 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.

2.1.9. Reagents and kits for Syber green quantitative real time PCR: IFγ gene Primers were used according to (Rafael *et al.,* 2011) as shown in table 1.

Table 1: IFγ gene analysis primers:

Primer	Sequence	No. of bases
If γ FOR	5-CGGCACAGTCATTGAAAGCCTA-3	22
If γ REV	5-GTTGCTGATGGCCTGATTGTC-3	21

2.1.10. SYBER Green qPCR kit: It was obtained from (Fermentas, Cat. No K0259) for 60 reactions of 25 µl. This Kit cosists of:

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

2.1.11. Real time PCR machine: Stratagen MX3005P machine (Stratagen, USA).

2.2. Methods

2.2.1. Titration of RVF virus: The virus was titrated in tissue culture as well as in mice:

A. In Tissue Culture: BHK_{21} cells were grown and maintained according to Macpherson and Stocker (1962). Ten fold serial dilutions of the of the seed virus were prepared in hank's solution and inoculated into confluent BHK_{21} cell culture 10 µl per well and 3 wells per each dilution. The virus was allowed to be adsorbed by incubating the cultures for one hour at 37ºC followed by addition of maintenance media and reincubated at 37ºC with daily observation for CPE for a period 5-7 days. the titer was expressed as $TCID_{50}$/ 0.1ml. Of the original inoculum using the formula of Reed and Muench 1938.
B. In Mice: 21-30 days old mice (10 mice/ each dilution) were used for this purpose. Serial ten fold dilutions of the virus to be titrated were prepared. 10 adult mice were used for each dilution including a control

group of non infected mice. Each mice was intraperitoneally inoculated (I /P) with 0.2 ml of the virus daily recorded for a period of 10 days from the 2nd day post inoculation (those dying in the first 24 hours were excluded and considered non- specific death). The virus titer was expressed as log $_{10}$ MIPLD$_{50}$ / 0.2 ml of the original inoculum according to the formula of Reed and Muench (1938).

2.2.2. Vaccine preparation: Procedure of vaccine production including the following steps:

A. Inactivation process: The RVF virus was inactivated with Binary (2-Bromoethyle ammonium bromide with sodium hydroxide) according to Black burn and Besselaar (1991) to neutralize the residual BEI and stop its overreaction.

B. Preparation of batches of RVF vaccine: Preparation of 2 batches of inactivated RVF vaccine: batch 1 using Aluminum hydroxide gel as adjuvant with a concentration of 20% and batch 2 using montanide gel as adjuvant with concentration as 20%.

C. Evaluation of the prepared batches of vaccine:

Sterility test: The prepared inactivated RVF vaccines were tested for its sterility.

Potency test: Adult mice (21-28 days old) were inoculated I/P by 2 doses of vaccine, one week apart, and then challenged to calculate the ED$_{50}$ according to Reed and Muench (1938).

2.2.3. Monitoring the validity of the prepared inactivated RVF vaccines: By estimating the shelf life of 20% montanide and Aluminum hydroxide gel inactivated RVF vaccine at room temperature and at 4˚ C by applying potency test with intervals of 1st month, 2nd month, 3rd month, 6th month, 10th month, 11th month and 12th month of the prepared vaccines.

2.2.4. Evaluation of cell mediated immune response in mice:

2.2.4.1.Challenge test: Three weeks old SPF mice obtained from breeding unit, veterinary serum and vaccine research institute, Abbassia, Cairo, Egypt were divided into four groups each group of 15 mice as in table (2).

A. Separation of mice blood lymphocytes: It was performed according to (Boyum, 1968; Lucy, 1977; and Lee, 1984)

B. Counting of viable cell number: it was performed according to Hudsom and Hay, (1980):

C. Interferon γ (If γ) gene analysis by SYBER Green quantitative real time PCR for mice:

- *Total RNA extraction from mice lymphocytes using Trizol (Invitrogen, Carlsbad, CA):* according to *Simms et al., 1993.*
- *1st strand cDNA synthesis:* Reaction mix volumes / one reaction using 1st strand cDNA synthesis by Access quick RT-PCR (promega):
- *SYBER Green quantitative real time PCR:* By using Maxima™ SYBR Green qPCR master mix (2X) (Fermentas, Canada)

3. RESULTS

1. Propagation and titration of RVF virus (ZH501):

Three batches of RVF virus vaccine and titrated in both adult mice and in tissue culture. The highest titer was chosen for vaccine preparation (Tab. 3).

2. Safety test of inactivated virus:

The inactivated RVFV did not express any CPE in inoculated BHK cells, or mortality or any signs of illness (allergic reaction, inflammation, granuloma, swelling, and sterile abscess) were observed on inoculated suckling mice during the 10 days of the observation period.

3. Toxicity of 20% montanide gel adjuvant in adult mice:

There was no toxic effect .

4. Quality control of the prepared vaccine:

A. Sterility test:

Table 2 : Groups.

Mice Groups	No. of mice	Treatment	challenge
1	15	Vaccinated with 20%Aluminium hydroxide gel inactivated RVF vaccine	Challenged with 0.1 ml of 10^4 $MIPD_{50}$ /ML RVF virus.
2	15	Vaccinated with 20% montanide gel inactivated RVF vaccine	Challenged with 0.1 ml of 10^4 $MIPD_{50}$ /ML RVF virus.
3	15	Non vaccinated and challenged (+ve control)	Challenged with 0.1 ml of 10^4 $MIPD_{50}$ /ML RVF virus.
4	15	Non vaccinated non challenged (-ve control)	Not challenged

Table 3: Titration of RVFV (ZH 501):

Batch No.	Titer in adult mice expressed as log_{10} $MIPLD_{50}$/ml	Titer in TC expressed as log_{10} $TCID_{50}$/ml
1	$10^{6.5}$	$10^{7.3}$
2	$10^{7.2}$	10^{8}
3	$10^{7.5}$	$10^{8.5}$

$MIPLD_{50}$: Mice intraperetoneal leathal dose 50.
$TCID_{50}$: Tissue cultur infective dose 50.

Table 4: Shelf life of the inactivated RVF vaccines:

Type of vaccine	Time (month)									
	0	2	4	6	7	8	9	10	11	12
Aluminum hydroxide vaccine	0.0017	0.0015	0.0019	0.002	0.0026	0.0038	0.0091	0.020	0.032	0.051
Montanide gel vaccine	0.0011	0.0019	0.002	0.0024	0.0036	0.0041	0.0062	0.0082	0.010	0.017

Table 5: The mean value of lymphocytic count of mice groups vaccinated with aluminum hydroxide and montanide gel vaccines and challenged after with RFV virus

Vaccinated Mice groups / Time	Group vaccinated by Aluminum hydroxide vaccine	Group vaccinated by Montanide Gel vaccine	Control positive	Control negative
24 hr before challenge	19.8*	36.6	13.6	13.5
24 hr after challenge	16.2	27	34.6	12.8
48 hr after challenge	19.8	37	38.5	13.3
72 hr after challenge	8.8	16.1	11	13.5

***:** lymphocytic count $X10^5$/ml.D_{50}: Effective dose 50

Table 6: Interferon γ gene expression in mice vaccinated with different types of inactivated RVF vaccine and challenged after by RVF virus (expressed by cycle threshold) (ct)

Mice groups / Time	Aluminum hydroxide vaccine	Montanide Gel vaccine	Control positive	Control negative
24 hr before challenge	36.43	30.55	-	No ct
24 hr after challenge	37.05	35.70	No ct	-
48 hr after challenge	34.93	32.01	33.59	-
72 hr after challenge	34.35	30.66	30.79	-

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

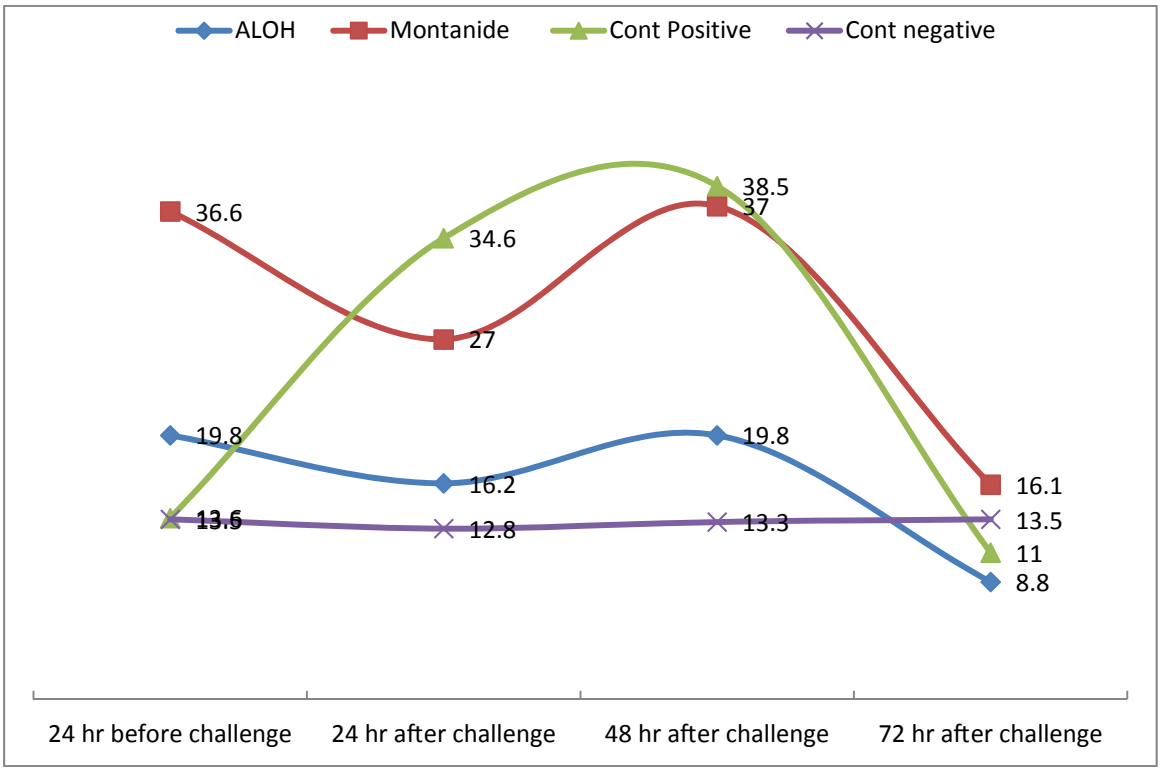

Fig. 1: The average number of viable mice lymphocytes / ml of different groups of mice vaccinated with 2 types of inactivated RVF vaccines and challenged with RVF virus with relation to the time of blood sample collection.

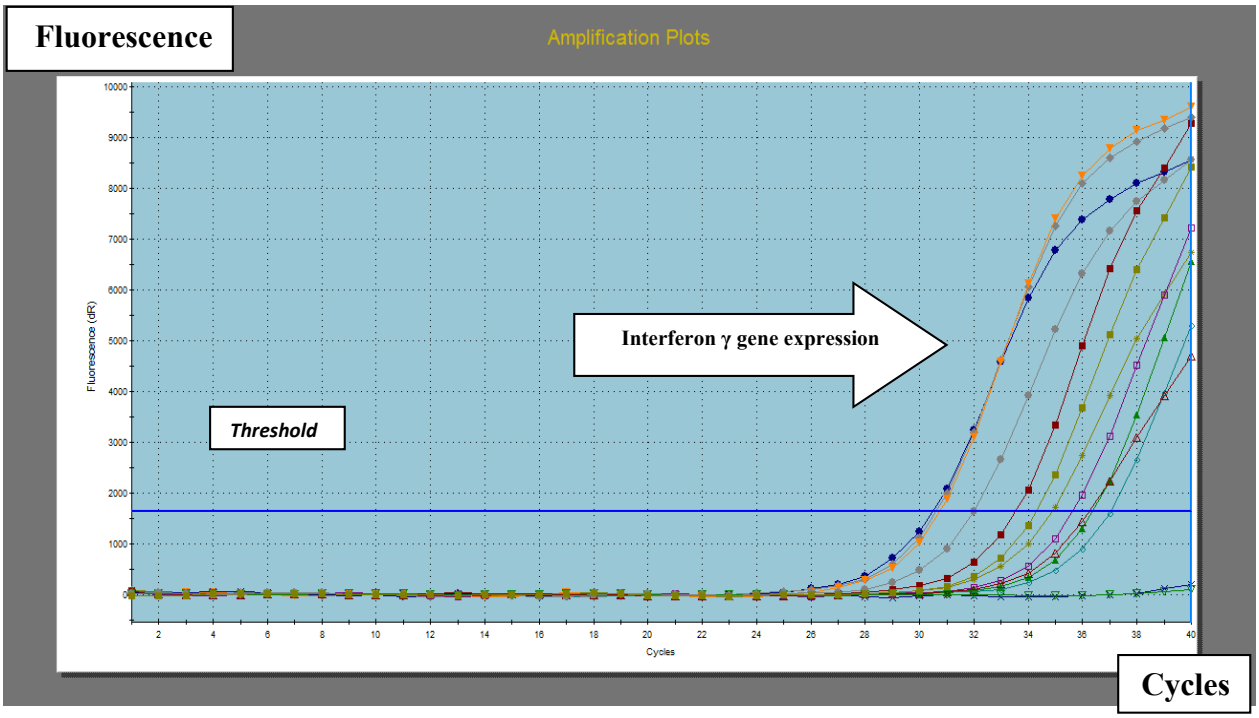

Fig. 2: Interferon γ gene expression in mice vaccinated with montanide gel and aluminum hydroxide inactivated RVF vaccines and challenged by RVF virus.

The results showed that no bacterial, fungal or mycoplasmal growth was observed during the period of observation (14 days).

B. Potency test in mice:

Both vaccines gave an acceptable ED50

5. Shelf life (keeping quality):

The results showed that 20% montanide gel vaccine was within the permissible limit (0.02 ED_{50}/ ML) for 12 months while the aluminum hydroxide gel vaccine still up to 10 months only.

6. Lymphocytic count of mice vaccinated with the inactivated RVF vaccines and challenge:

The mean value of lymphocytic count showed that there was a highly significant increase in lymphocytic count in the mice group vaccinated with montanide gel vaccine at 24 hours before challenge compared to that vaccinated with aluminum hydroxide vaccine (Tab. 5 and Fig. 1). Similar results were obtained at 48 hours after challenge where there were highly significant increase in lymphocytic count in the mice group vaccinated with montanide gel vaccine and the control positive group (challenged non vaccinate group) in the comparison with the group vaccinated with aluminum hydroxide vaccine .

7. Detecting interferon γ gene expression in blood of mice using Syber green quantitative real time PCR:

The results revealed that mice vaccinated with montanide gel inactivated vaccine and boostred at one week apart showed high level of interferon γ gene expression 24 hours before challenge. In other hand, after challenge with 10^3 $TCID_{50}$ RVF virus, the interferon γ gene expression was increased gradually till reach high level in the 3rd day after challenge when compared with the level of interferon γ in mice vaccinated with Aluminum hydroxide vaccine. The results showed no expression for interferon γ gene in the negative control group. While in the positive control group, the interferon γ gene expression increased gradually till reach high level in 3rd day after challenge (Tab. 6 and Fig. 2).

4. DISCUSSION

The progress in vaccine production is directed towards the selection of the proper adjuvant that can elaborate high and long standing immunity. Adjuvant is considered one of the important factors in vaccine formulation as it can influence the immune response referred to vaccine. Therefore, this study is applied to develop an inactivated RVF vaccine with 20% montanide oil as adjuvant and evaluate the immune response and duration of immunity in mice with the comparison with the aluminum hydroxide inactivated vaccine. The used virus in vaccine preparation was titrated d in BHK tissue culture and the titer was equal $10^{8.5}$ $TCID_{50}$/ ml. Then the virus was inactivated by using binary ethylenamine inactivator according to *Eman (1995).* The inactivated virus safety was tested using tissue culture and suckling mice. The results revealed that did not show any CPE on tissue cultures and any clinical signs. Toxicity of the montanide gel adjuvant were tested by I/P inoculation of adult mice, results revealed that the 20% concentration of montanide gel were non toxic during 10 days of observation. Results of evaluating the two types of binary inactivated vaccines by testing their sterility showed that they were free from any bacterial, fungal and mycoplasma contaminations. The prepared vaccines were safe when inoculated into mice with no deaths or any symptoms denote RVF infection due to residual infectivity virus on the prepared vaccine.

Results of potency of the prepared vaccines in adult mice revealed that the vaccines gave a protection in mice with ED_{50}/ml (0.0017, and 0.0011 for the aluminum hydroxide gel and montanide gel inactivated vaccines, respectively). The shelf life of both types of the vaccines was tested for 12 months at 4°C. The montanide gel vaccine was within the permissible limit (0.02 ED_{50}/ML) up to 12 months. This result agreed with that reported previously by *Randall et al., (1962)* and *Gehan et al., (1998).* While the aluminum hydroxide gel vaccine was still up to 10 months only. The results obtained similar to results of *Gihan et al., (1998)* who reported that the binary inactivated RVF vaccine with aluminium hydroxide gel was potent till 10 months when kept at 4°C.

The results of lymphocytic count revealed that mice vaccinated with montanide gel inactivated vaccine showed higher level of lymphocytic count 24 hr before challenge (7.3) and reached to the maximum level at 48 hr after challenge (7.4) with a significant higher level when compared with lymphocyte count in mice vaccinated with aluminum hydroxide vaccine. These results agreed with that obtained by *karim et al., (1998)* who reported that lymphocytic count was significantly increased in sheep after vaccination with inactivated RVF vaccine. The results of this study cleared that the montanide gel inactivated vaccine expressed earlier and higher value of lymphocytic count than the aluminum hydroxide inactivated vaccine.

The level of interferon γ gene expression was detected in mice vaccinated with montanide gel inactivated vaccine with a booster dose 1 week apart and compared the results that obtained in mice vaccinated with aluminum hydroxide inactivated vaccine with booster dose also 1 week apart. The results revealed that montanide gel induced higher level of interferon γ gene expression than aluminum hydroxide gel.

The data of this study clearly showed that the montanide gel was highly immunogenic at concentration of 20% inducing a higher lymphocytic count and interferon γ level. Therefore, the montanide gel can be used as adjuvant at concentration 20% in preparation of inactivated RVF vaccine to get a better control of the disease among animals.

5. REFERENCES

Abdel-Gaffar, S., Mohsen, A., Ayoub, N., El-Nimr, M., Fathia, M. 1979. Rift Valley fever in Egypt III. Diagnosis and vaccination. 14th Arab. Vet. Cong., 24-29 march Cairo, page 1-11.

Blackburn, N.K., Besselaar, T.G. 1991. A study of the effect of chemical inactivants on the epitopes of Rift Valley fever virus glycoproteins using monoclonal antibodies. J Virol Methods. 1991 Aug;33(3):367-74.

Boyum, A. 1968. Isolation of mononuclear cells and granulocytes from human blood. Scand. J. Clin. Invest., 21: 77-89.

Digoutte, J.P., Peters, C.J. 1989. General aspects of the 1987 Rift Valley fever epidemic in Mauritania. Res. Virol.,140: 27-30.

Easterday, B.C., Murphy, L.C., Bennett, D.G. 1962. Experimental Rift Valley Fever in lambs and sheep. Am. J. Vet. Res., 23: 1231-1240.

Eman. M.S.S. 1995. Studies on RVF vaccine inactivated with Binary. Ph. D. thesis (Microbiology), Fac. of Vet. Med. Cairo -Univ.

Gihan, K.M., Elian, K.A., Eman, M.S. 1998. Studies on the keeping quality of binary inactivated Rift Valley fever Vaccine. Assiut Vet. J., 39 (77): 169-179.

Hudson, L., Hay, F.C. 1980. Practical Immunology. 2nd Ed., Blackwell Scientific Publication, Oxford, London, Edinburgh, Boston, Melbourne.

Lee, L.F. 1984. Proliferative response of chicken B and T lymphocyte to mitogen.Vet. Med., 15: 44-52.

Lucy, F.L. 1977. Chicken lymphocyte stimulation by mitogenes .A microassay with whole blood cultures. Avian Dis., 22: 296-307.

Macpherson, J.A., Stocker, N.G. 1962. Polyoma transformation of hamster cell clones an investigation of genetic factors affecting all competence. Virology, 16: 147-151.

OIE 1989 Manual of recommended diagnostic techniques and requirements for biological products of lists A and B diseases. International Committee of the OIE, 2: 1-8.

Paweska, J.T., Burt, F.J., Anthony, F., Smith, S.J., Jrobbelaar, A.A., Croft, J.E., Ksiazek, T.G., Swanepoel, R. 2003. IgG sandwich and IgM capture ELISA for the detection of antibody to RVFV domestic ruminants. Virol. Methods, 113 (2): 103-112.

Rafael, F. Botrán, Zeshan A., Fabián, A. Crespo, Chandler, G., John, G., Dennis, W.D., Irene, L. 2011. Cytokine expression and microglial activation in progressive supranuclear palsy.parkinsonism relat disord. Nov. 2011; 17(9): 683–688.

Randall, R., Gibbs, C.J., Aulisio, C.G., Sinn, L.A., Harrison, V.R., 1962. The development of a formalin-killed Rift Valley Fever vaccine for use in man. J. Immun., 89 (5): 660-671.

Reed, L.J., Muench, H. 1938. A simple method for estimating 50 percent end point. Am. J. Hyg., 27: 493-497.

Simms, Domenica, Paul, E.,Cizdziel, Piotr, C.N. 1993. Trizol:Anew reagent for optimal single step of RNA. Focus 15.4(1993):532-535.

التحصين ضد مرض حمى الوادي المتصدع

ديانا محمد أبو المجد[1]، محمد حسنين عبيد[2]، عبد المنعم مصطفى[2] ، ايمان محمد سيد شلقامي[1], محمد جوده عبد الوهاب[2]
[1]وحدة حمى الوادي المتصدع-معهد الامصال واللقاحات بالعباسية-القاهرة، [2]الأمراض المعدية-قسم طب الحيوان—كلية الطب البيطري-جامعة بنها

الملخص العربي

في هذه الدراسة تم تحضير لقاح حمى الوادي المتصدع باستخدام المونتانيد جل 20% بالإضافة الى اللقاح المستخدم محليا بالألومنيوم هيدروكسيد جل للمقارنة وتناولت الدراسة اجراء اختبار الامان لكل لقاح على حده على الفئران ولم يتسبب اي منهما في حدوث اعراض مرضيه او نفوق. كما تم قياس كفاءة اللقاحين على الفئران كانت النتائج كالتالي (0.0017 و 0.0011 ML/ ED_{50}) للقاح الالومنيوم هيدروكسيد جل ولقاح المونتانيد جل على التوالي، ثم بعد ذلك تم تقييم هذه الكفاءة على مدار عام مع حفظ اللقاحين عند درجة حراره 4) (°Cوكان لقاح المونتانيد جل الاكثر كفاءه على طول مدة التجربة ولم يتعدى الحد الاقصى 0.02 ML/ED_{50} . كذلك تم تقييم المناعة الخلوية في الفئران المحصنة بجرعتين من لقاح حمى الوادي المتصدع باستخدام المونتانيد جل 20% بفاصل اسبوع ثم تم اعطائه جرعة من الفيروس الضاري لحمى الوادي المتصدع 1 مل تحت الجلد للتحدي ومقارنتها بالمجموعة المحصنة بلقاح حمى الوادي المتصدع باستخدام الالومنيوم هيدروكسيد جل وذلك من خلال قياس عدد الخلايا (تي) في الدم، وقد وجد ارتفاع سريع واعلى في الفئران المحصنة بلقاح المونتانيد جل.
وفي نفس إطار هذه التجربة تم ايضا قياس مستوى الانترفيرون جاما وذلك عن طريق تحديد مستوى الجين المعبر عنه في الدم، ووجد في المجموعة المحصنة بلقاح حمى الوادي المتصدع باستخدام المونتانيد جل، أن الجين المعبر عن الانترفيرون جاما أعلى من المجموعة المحصنة بلقاح الالومنيوم هيدروكسيد جل. ومن هذا يمكننا القول بان لقاح حمى الوادي المتصدع باستخدام المونتانيد جل 20% هو الافضل بسبب ما ينتج عنه من ارتفاع واضح في مستوى المناعة الخلوية على طول مدة التجربة.

(مجلة بنها للعلوم الطبية البيطرية: عدد 27(1) ،239 -247: سبتمبر 2014)