

Immunomodulin Effect of Inactivated Parapox Virus (Zylexis) on Sheep Crop Vaccinated with Inactivated RVF Vaccine

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Abstract

Rift Valley Fever (RVF) is a mosquito-borne zoonotic disease that presents a substantial threat to human and public health. It is caused by Rift Valley fever *Phlebovirus* (RVFV), which belongs to the genus *Phlebovirus* and the family *Bunyaviridae*. Both wide distribution of the vectors in non-endemic areas and the climatic change, cause great threat of RVFV spreading.

So, the present study was used zylexis as immunomodulatory factor to increase the immune response of sheep vaccinated with inactivated RVFV vaccine. Twenty sheep were divided into four groups (five animals/group) where the first group was inoculated with Zylexis (2 ml/sheep) two days before vaccination with the field dose 1 ml, (s/c) of inactivated RVF vaccine; the second group was inoculated with Zylexis simultaneously with the vaccine; the third sheep 'group was inoculated with the vaccine only while the fourth group was kept as non-vaccinated and non-inoculated control. Blood samples were collected on heparin as anticoagulant for evaluation of cellular immune response, while serum samples had been collected for monitoring the humeral immune response using SNT and ELISA. The obtained results demonstrated that sheep inoculated with zylexis with the inactivated RVF vaccine give more humoral and cellular immune response than those inoculated with zylexis two days before vaccination and those inoculated with the vaccine only. These findings revealed that zylexis acts as an immune stimulator in sheep vaccinated with the inactivated RVF vaccine.

Keywords: RVF; SNT; ELISA; Zylexis

Introduction

Rift Valley fever virus (RVFV) affects humans and livestock in sub-Saharan Africa, Egypt, Yemen and Saudi Arabia. Since the first outbreak in Kenya in 1931 [1], recurrent outbreaks caused deaths of hundreds of thousands of animals, more than a thousand humans, and great economic losses [2,3]. Regarding animals, RVFV is mainly transmitted by a range of mosquito species (*Aedes*, *Anopheles*, *Culex*, *Eretmapoites*, *Mansonia*), but has also been shown to be transmitted by other vectors e.g. sandflies [4,5]. Infected animals suffered from necrotic hepatitis, hemorrhage and abortion, with mortality rates reached to 100% among newborn animals [3]. Humans infection occurred by close contact with sick animals [6,7]. The disease's symptoms in humane ranged from uncomplicated acute febrile illness to retinitis, hepatitis, renal failure, meningoencephalitis, conjunctivitis with ocular complications, severe hemorrhagic disease and death [3,8]. Although the mortality rate for humans was reported to be approximately 2%, in recent outbreaks it went up to 45% [9,10]. The severity of RVFV zoonosis caused by its ability to cause epidemics among livestock and humans, and the deficiency of efficient prophylactic

and therapeutic measures. So, the infection with this pathogen as serious public health concern not only in endemic developing countries, but also in many non-endemic industrial countries.

The importation of infected ruminants and camels from Sudan [11] causes introduction of the disease to Egypt and the reappearance occurred in 1993 in Egypt [12]. The vector control and vaccination are the best methods for protection of animal population and indirectly human beings [13]. There are two types of RVF vaccine live attenuated Smithburn and inactivated vaccine [14] but using of it is limited is due to teratogenic and abortogenic effect [15].

It is well known that vaccination is the corner stone in control of viral diseases. In Egypt, inactivated RVF vaccine is used to provide them with the required protective level of specific immunity for a suitable period of immunity duration. Such purpose could be achieved by the use of an immune modulating agent. Zylexis is a para-immunity activator containing inactive parapoxvirus ovis which used as a protective agent against different infectious diseases [16].

The paramunity inducer zylexis (PIND-ORF) is reported to stimulate the innate immune system. Paramunity can be defined as acquired non-pathogen-specific and non-antigen-specific protection of limited duration against adversity of noxious influences, such as foreign substances, infectious pathogens, toxins, and malignant cells. Paramunity inducers are non-immunizing biological products with a paraspecific effect on the innate immune system [17]. Paramunity inducers can enhance the rate of phagocytosis by stimulating monocytes and macrophages, boosting the function of spontaneous cell-mediated cytotoxicity by natural killer (NK) cells and improving the activity of other lymphoreticular cells. They also can enhance production, release, and interaction of many cytokines, such as IFN- α and IFN- γ , interleukin (IL)-2, tumor necrosis factor (TNF)- α and granulocyte macrophage colony stimulating factor (GM-CSF) [18]. In some studies, it has been reported to be effective when used metaphylactically or therapeutically [19-23].

To obtain high protective immune levels through application of well designed vaccination program; the use of an immune modulator agent may be required. So, the present work highlights the effect of Zylexis on the immune response of sheep to the inactivated RVF vaccine.

Materials and Methods

Rift valley fever virus (RVFV 'ZH 501')

It was obtained from RVF Department, Veterinary Serum and Vaccine Research Institute (VSVRI). With a titer of $10^{7.5}$ TCID₅₀/ml. This virus was used for serum neutralization test (SNT).

Tissue culture

African green monkey kidney cell line (Vero Cells) was grown and maintained according to [24] and was used for virus propagation, titration and serum neutralization test. It was obtained from RVF department.

Na heparin

It was used as anticoagulant in sterile plastic centrifuge tube (200 IU/ml) used for collection of blood for lymphocytic proliferation assay, it was obtained from Central Laboratory for Evaluation of Veterinary Biologics, Abbasia, Cairo.

Zylexis

Inactivated Parapox Ovis virus strain D107 (commercially known as zylexis - Zoetis, Animal Health) was used to enhance the immune

response of sheep to the inactivated RVF vaccine using a dose of 2 ml/animals according to [25].

Inactivated RVF vaccine

It was supplied by RVF department and used for vaccination of experimental sheep.

Experimental animals

- **Adult mice:** 21 - 30 days old Swiss albino weaned mice and specific pathogen free, were supplied by the Breeding Unit, (VSVRI) and it was used in ED₅₀ potency test.
- **Newly born lamb:** Five apparently healthy newly born lambs (7 - 10 days old) were used for safety test of inactivated RVF Vaccine; it was supplied by Breeding Unit, (VSRI).
- **Sheep:** Twenty local healthy breed sheep 3 - 4-month-old and free from external and internal parasites were used in the present work. All of these animals were proved to be free from RVF antibodies using SNT and ELISA and housed under strict hygienic measures in insect-proof stables receiving balanced ration and adequate water.

Positive and negative control sera

Positive and negative anti-RVF sera were supplied by RVF department to be used in SNT and ELISA.

ELISA kits

ELISACat. No. 3340 was obtained from COSTER 205 Broadway Cambridge, MA 02139.

Vaccine quality control

- **Sterility test:** Samples were taken from the vaccine as well as the virus fluid before inactivation process and tested on Nutrient agar medium, Sabouraud dextrose agar medium, Thioglycolate medium (Oxford, England) and PPLO (broth) medium for detection of bacterial fungal and mycoplasma contaminations [26,27].
- **Safety test:** Three lambs were inoculated with 10 ml of prepared inactivated vaccine (five ml S/C and five ml I/P), while two was kept as control. Lambs were observed daily till two weeks for any rise of temperature, death and any abnormal clinical signs post vaccination.
- **Potency test (ED₅₀):** In adult mice it was applied according to [28].

Experimental design

The twenty sheep were divided into four groups (five animals/group) as follow:

- **Group (1):** Injected I/M with 2ml/animal of Zylexis two days before vaccination with inactivated RVF Vaccine.
- **Group (2):** Inoculated with the same dose of Zylexis simultaneously with inactivated RVF vaccine.
- **Group (3):** Inoculated with inactivated RVF vaccine only

- **Group (4):** Was kept without any injections as negative control.

Collection of serum and blood samples

The serum samples were collected at weekly intervals in the first month then monthly till the end of experiment period (40 weeks post vaccination) for serological tests (SNT, ELISA), while blood samples were collected at 0, 3, 7, 10, 14, 21 and 28 days post vaccination for evaluation of cellular immunity.

Evaluation of the humoral immune response

Serum neutralization test (SNT)

This test was used for monitoring of the specific induced RVF neutralizing antibodies in vaccinated sheep. The serum-neutralizing index was calculated according to [29].

Indirect enzyme-linked immunosorbent assay (ELISA)

It was carried out according to [30] and the cut off value is calculated according to [31].

Evaluation of cell-mediated immune response

Lymphocyte blastogenesis using XTT assay

The cleavage of the tetrazolium salt (3-[4, 5- dimethyl thiazole-2yl]-2,5 -diphenyltetrazoliumbromide) into a blue colored product (formazan) by the mitochondrial enzyme succinate dehydrogenase [32] 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: According to [33,34].
- ii) Setting up of lymphocyte and using cell proliferation kit (XTT kit) according to [35].

Statistical analysis

The obtained data were statistically analysed by (SPSS system, version 16) to estimate mean \pm standard error (ST) and ANOVA for the effects of treatment and time on parameters under investigation [36].

Results

Propagation of RVF virus (ZH501) in cell culture

The virus was propagated in Vero cells. A prominent CPE of RVF virus in Vero cells appeared as rounding and aggregation in clusters (grapes like aggregation) as shown in photo 2. Such description of came in complete agreement with what described before by [37].

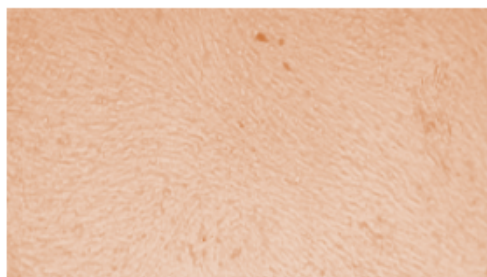


Photo 1: Normal Vero cells.



Photo 2: CPE of RVFV on Vero cells.

Quality control of prepared vaccine:

- **Sterility:** Inactivated RVF vaccine gave satisfactory results. It was free from mycoplasma, aerobic, anaerobic bacteria and fungi.
- **Safety test:** Lambs showing no rise in body temperature with no post vaccinal reaction and any abnormal signs.
- **Potency test of prepared vaccine in mice (ED50):** The inactivated RVF vaccine gave 0.0025 ED₅₀/ ML (permissible limit less than 0.02/ml).

Evaluation of cellular immunity

Sheep group	Mean optical densities of lymphocytic proliferation assay\DPV*								Overall mean
	Zero	3 rd	5 th	7 th	10 th	14 th	21 st	28 th	
Gp (1)	0.17 ± 0.08	0.66 ± 0.09	0.62 ± 0.11	0.69 ± 0.07	0.60 ± 0.12	0.56 ± 0.09	0.40 ± 0.12	0.35 ± 0.12	0.506(b) ± 0.08
Gp (2)	0.16 ± 0.11	0.78 ± 0.09	0.82 ± 0.09	0.92 ± 0.08	1.03 ± 0.08	1.33 ± 0.09	0.98 ± 0.09	0.86 ± 0.09	1.61(a) ± 0.07
Gp (3)	0.17 ± 0.08	0.41 ± 0.07	0.47 ± 0.07	0.54 ± 0.08	0.58 ± 0.09	0.43 ± 0.11	0.30 ± 0.08	0.15 ± 0.08	0.381(c) ± 0.08
Gp (4)	0.13 ± 0.09	0.09 ± 0.08	0.11 ± 0.08	0.1 ± 0.07	0.12 ± 0.09	0.11 ± 0.08	0.08 ± 0.06	0.12 ± 0.08	0.107(e) ± 0.08

Table 1: Lymphocytic blastogenesis assay in different sheep groups inoculated with inactivated RVF vaccine and Zylexis.

DPV*: Days Post Vaccination.

Gp (1) inoculated with zylexis 2 days before vaccination.

Gp (2) inoculated with zylexis at time of vaccination.

Gp (3) inoculated with inactivated RVF vaccine.

Gp (4) non-vaccinated control.

Sheep group	Phagocytic percentage (%) / DPV*								Overall mean
	Zero	3 rd	5 th	7 th	10 th	14 th	21 st	28 th	
Gp (1)	17 ± 0.08	44 ± 0.08	48 ± 0.09	56 ± 0.11	54 ± 0.07	52 ± 0.07	48 ± 0.11	40 ± 0.08	45(b) ± 0.08
Gp (2)	19 ± 0.11	62 ± 0.07	65 ± 0.08	72 ± 0.09	78 ± 0.11	80 ± 0.08	74 ± 0.07	68 ± 0.07	65(a) ± 0.07
Gp (3)	20 ± 0.07	27 ± 0.08	32 ± 0.07	36 ± 0.07	34 ± 0.07	31 ± 0.08	28 ± 0.08	23 ± 0.11	29(c) ± 0.09
Gp (4)	21 ± 0.08	21 ± 0.07	18 ± 0.09	19 ± 0.08	20 ± 0.09	17 ± 0.11	19 ± 0.07	17 ± 0.07	19(d) ± 0.07

Table 2: Phagocytic percentage obtained in different sheep groups inoculated with inactivated RVF vaccine and Zylexis.

DPV*: Days Post Vaccination.

Gp (1) inoculated with zylexis 2 days before vaccination.

Gp (2) inoculated with zylexis at time of vaccination.

Gp (3) inoculated with inactivated RVF vaccine.

Gp (4) non-vaccinated control.

Sheep group	Phagocytic Index /DPV*								Overall mean
	Zero	3 rd	5 th	7 th	10 th	14 th	21 st	28 th	
Gp (1)	0.13 ± 0.07	0.31 ± 0.08	0.36 ± 0.09	0.44 ± 0.11	0.40 ± 0.07	0.38 ± 0.07	0.34 ± 0.11	0.30 ± 0.07	0.37(b) ± 0.08
Gp (2)	0.11 ± 0.07	0.41 ± 0.07	0.53 ± 0.08	0.60 ± 0.09	0.75 ± 0.11	0.84 ± 0.08	0.76 ± 0.07	0.68 ± 0.07	0.58(a) ± 0.07
Gp (3)	0.12 ± 0.07	0.25 ± 0.08	0.30 ± 0.07	0.33 ± 0.07	0.36 ± 0.07	0.33 ± 0.08	0.28 ± 0.08	0.14 ± 0.07	0.26(c) ± 0.09
Gp (4)	0.11 ± 0.07	0.13 ± 0.07	0.11 ± 0.09	0.12 ± 0.08	0.12 ± 0.09	0.12 ± 0.11	0.11 ± 0.07	0.11 ± 0.07	0.12(d) ± 0.07

Table 3: Phagocytic indices obtained in different sheep groups inoculated with inactivated RVF vaccine and zylexis.

DPV*: Days Post Vaccination.

Gp (1) inoculated with zylexis 2 days before vaccination.

Gp (2) inoculated with zylexis at time of vaccination.

Gp (3) inoculated with inactivated RVF vaccine.

Gp (4) non-vaccinated control.

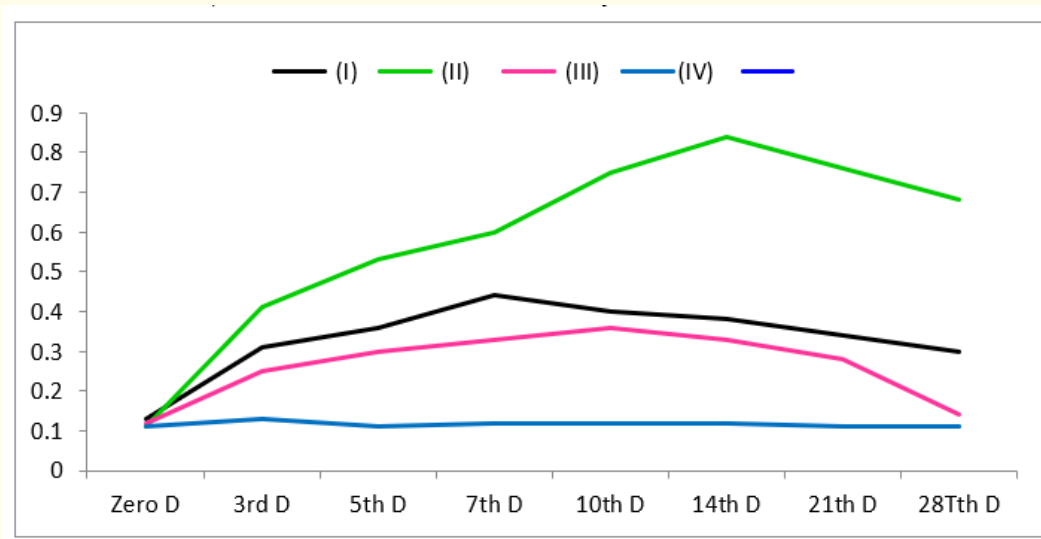


Figure 1: Lymphocytic blastogenesis assay of different sheep groups inoculated with inactivated RVF vaccine and Zylexis.

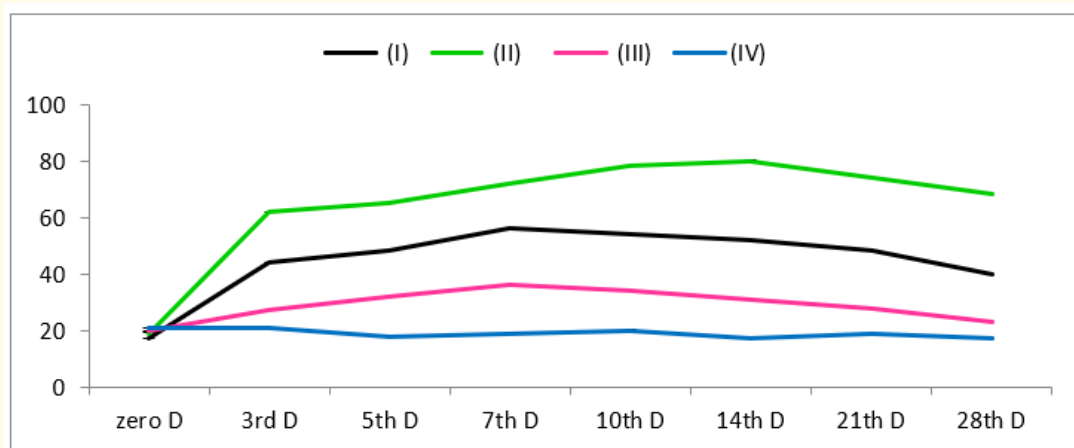


Figure 2: Phagocytic percentage obtained in different sheep groups inoculated with inactivated RVF vaccine and Zylexis.

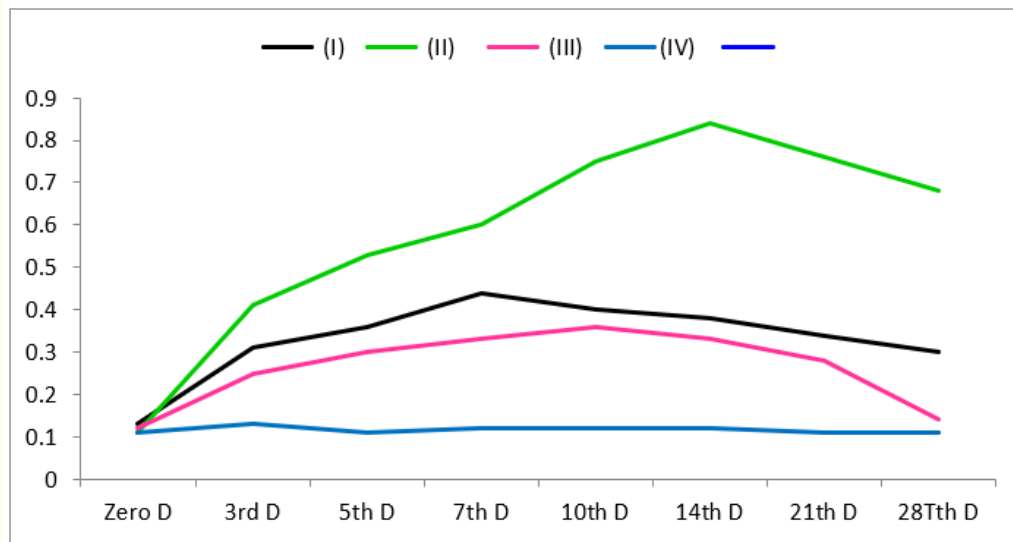


Figure 3: Phagocytic indices obtained in different sheep groups inoculated with inactivated RVF vaccine and zylexis.

Evaluation of the humoral immune response:

Animal group	Mean RVF neutralizing indices /WPV*													Over-all mean
	0 day	1 st	2 nd	4 th	6 th	8 th	12 th	16 th	20 th	24 th	32 nd	36 th	40 th	
Gp (1)	0.6 ± 0.09	1.00 ± 0.12	2 ± 0.08	2.57 ± 0.08	2.86 ± 0.09	3.1 ± 0.08	2.88 ± 0.12	2.7 ± 0.05	2.62 ± 0.08	2.2 ± 0.09	1.74 ± 0.09	1.39 ± 0.09	0.96 ± 0.09	2(b) ± 0.11
Gp (2)	0.9 ± 0.09	1.7 ± 0.02	2.2 ± 0.12	2.76 ± 0.08	3.03 ± 0.09	3.5 ± 0.07	3 ± 0.11	2.9 ± 0.15	2.77 ± 0.09	2.4 ± 0.04	1.92 ± 0.09	1.74 ± 0.06	1.2 ± 0.09	2.3(a) ± 0.08
Gp (3)	0.75 ± 0.09	0.90 ± 0.06	1.74 ± 0.06	2.46 ± 0.12	2.73 ± 0.05	2.9 ± 0.09	2.80 ± 0.06	2.63 ± 0.12	2.52 ± 0.07	2.00 ± 0.05	1.64 ± 0.12	1.30 ± 0.09	0.96 ± 0.12	1.9(c) ± 0.11
Gp (4)	0.6 ± 0.09	0.75 ± 0.08	0.9 ± 0.08	0.6 ± 0.11	0.75 ± 0.08	0.9 ± 0.07	0.8 ± 0.06	0.6 ± 0.07	0.9 ± 0.05	0.9 ± 0.06	0.75 ± 0.12	0.6 ± 0.11	0.9 ± 0.05	0.72(e) ± 0.02

Table 4: Mean RVF neutralizing indices of sheep inoculated with inactivated RVF vaccine and Zylexis.

*WPV: Week Post Vaccination.

Gp (1) inoculated with zylexis 2 days before vaccination.

Gp (2) inoculated with zylexis at time of vaccination.

Gp (3) inoculated with inactivated RVF vaccine.

Gp (4) non-vaccinated control.

Animal group	Mean ELISA optical densities/ WPV*													
	0 day	1 st	2 nd	4 th	6 th	8 th	12 th	.16 th	20 th	24 th	32 nd	36 th	40 ^h	Overall mean
Gp (1)	0.013 ± 0.001	0.160 ± 0.009	0.239 ± 0.001	0.260 ± 0.015	0.280 ± 0.008	0.314 ± 0.009	0.298 ± 0.007	0.280 ± 0.004	0.271 ± 0.007	0.263 ± 0.007	0.244 ±0.01	0.225 ± 0.006	0.200 ± 0.006	0.234(b) ± 0.11
Gp (2)	0.049 ± 0.009	0.231 ± 0.002	0.255 ± 0.002	0.288 ± 0.008	0.302 ± 0.009	0.334 ± 0.007	0.325 ± 0.006	0.310 ± 0.006	0.294 ± 0.009	0.281 ± 0.004	0.268 ± 0.009	0.249 ± 0.006	0.225 ± 0.009	0.262(a) ± 0.08
Gp (3)	0.022 ± 0.009	0.134 ± 0.006	0.235 ± 0.006	0.249 ± 0.012	0.270 ± 0.005	0.286 ± 0.009	0.277 ± 0.006	0.270 ± 0.002	0.265 ± 0.007	0.250 ± 0.005	0.239 ± 0.002	0.200 ± 0.009	0.160 ± 0.002	0.219(c) ± 0.11
Gp (4)	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	0.014(d) ± 0.02

Table 5: Mean ELISA optical density in sheep inoculated with inactivated RVF vaccine and Zylexis.

Cut off: 0.230.

*WPV: Week Post Vaccination.

Gp (1) inoculated with zylexis 2 days before vaccination.

Gp (2) inoculated with zylexis at time of vaccination.

Gp (3) inoculated with inactivated RVF vaccine.

Gp(4) non-vaccinated control.

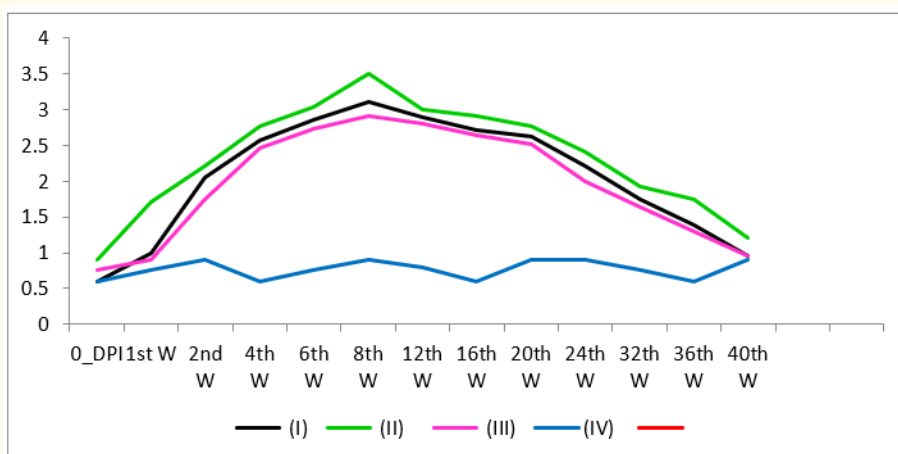


Figure 4: Mean RVF neutralizing indices of sheep inoculated with inactivated RVF vaccine and Zylexis.

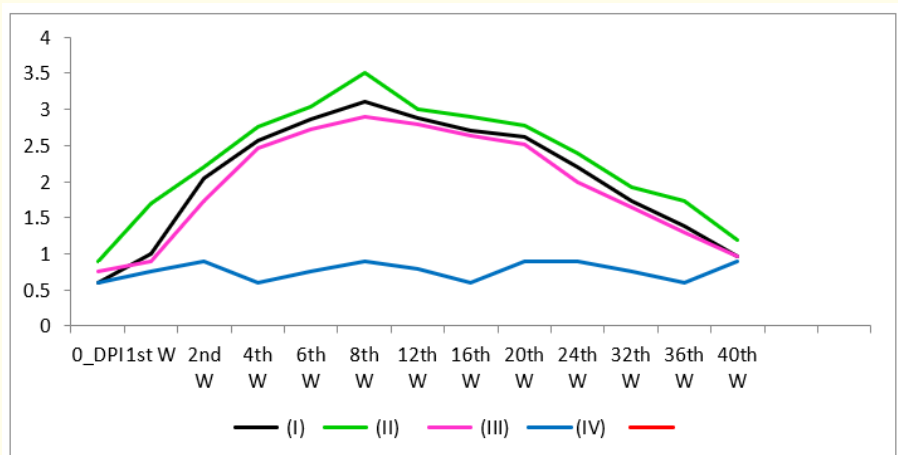


Figure 5: Mean ELISA optical density in sheep inoculated with inactivated RVF vaccine and Zylexis.

Discussion and Conclusion

RVF is an arthropod born viral zoonotic disease that affects domestic animals (including cattle, sheep, camels and goats) and human. The RVF virus is highly contagious for humans when come in contact to the infected animals or materials. 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 [38].

Good vaccination plane is very important way to control the disease. This study explained the important use of an immunomodulator (Zylexis) to improve the immunity by producing different immunomodulatory proteins that support active immune response.

Ulgen S., *et al.* [25] mentioned that Zylexis is currently used in equine medicine where it has a supportive effect on their cellular immunity and an immunomodulatory effect against equine viral infections. Such activity is based on the activation of innate cells and consequent cytokine production [39,40] reported that administrations of Zylexis as immunomodulator agent potentiate the cellular and humoral immune response and enhance the duration of immunity induced by the inactivated gel adjuvant Pneumo-4 vaccine.

Photo 1 showed propagation of RVFV viruses on VERO cell lines. The virus titers were expressed as the \log_{10} TCID₅₀/ml as described by [29]. It demonstrated the cytopathic effect which was characterized by rounding and aggregation of cells in clusters (grapes like appearance).

Table 1-3 and figure 1-3 showed that the use of Zylexis at the same time of vaccination with inactivated RVF vaccine give higher cellular immune responses in inoculated sheep than in those inoculated with Zylexis two days before vaccination. Cell proliferation assay, phagocytic percentage and phagocytic index readings recorded the peak values at 7th DPV (0.69, 56 and 0.44) for group-1 and at 14th DPV (1.33, 80 and 0.84) for group-2 and persisted up to 28 DPV (0.35, 40 and 0.30) in group-1 and (0.86, 68 and 0.68) in group-2. The results were agreeable with those of [18,25,40] whom reported that the immunomodulator activate innate immune cells.

The humeral immune response was evaluated using SNT as shown in table 4) and figure 4 showing that in gp. (1) there was an increasing of the neutralizing index (NI) started from two weeks post vaccination (2) to reach its peak at the 8th week post vaccination (3.1), while in gp (2) the (NI) started from 1 WPV (1.7) and reached the peak at the 8 WPV (3.5). The great elevation was in groups (1) and (2) (that receiving zylexis) than non-immune modulated group (3). These results come in agreement with those of [40] who reported that the level of the immune response in calves immune modulated with Zylexis is higher than in other non-immune modulated group. Table 5 illustrated that ELISA results of immune modulated sheep in group (1 and 2) are much higher than those of group (3), these results came in parallel to that obtained by SNT. It is cleared that administrations of Zylexis as immunomodulator agent enhance the cellular and humoral immune response and increase the duration of immunity induced by the inactivated RVF Vaccine. Kathryn A., *et al.* [17] reported that Paramunity inducers are non-immunizing biological products with a para specific effect on the innate immunity. They also added that Paramunity inducers enhance the rate of phagocytosis by stimulating monocytes and macrophages and improving the activity of other lymphoreticular cells. Such activity is based on the activation of innate cells and consequent cytokine production [39,40] reported that administrations of Zylexis as immunomodulator agent potentiate the cellular and humoral immune response and enhance the duration of immunity induced by the inactivated gel adjuvant Pneumo-4 vaccine.

Depending on the present obtained results, it could be concluded that zylexis could be used to enhance the induction of RVF antibodies in vaccinated sheep, which could lead to long duration of immunity.

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