Research Article

Evaluation of Humoral Immune Response to Simultaneous Vaccination with Ephemerovirus Febris and Phlebovirus Riftense Vaccines

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Abstract | *Ephemerovirus febris* and *Phlebovirus riftense* formerly known as Bovine ephemeral fever (BEF) and Rift Valley fever (RVF), respectively, are insect-borne viral diseases that affect cattle, causing significant economic losses. The application of a promising vaccination strategy requires the utilization of safe and effective vaccines capable of successfully preventing and controlling these disastrous diseases. The current study explored the effect of simultaneous vaccination of cattle with BEF and RVF vaccines on the calves' humeral immune response. Four groups of BEF and RVF seronegative calves were used in this study; group 1 received inactivated BEF vaccine, group-2 received inactivated RVF vaccine, group-3 received BEF and RVF vaccines simultaneously, and group 4 served as a negative control. The humeral immune response was evaluated by beta serum neutralization test (SNT) and ELISA. BEF antibody titers were detected by SNT in the 1st-week post first vaccination as 6 (by detecting the endpoint of the serum that inhibit completely 100 TCID50) in the group 1 and 5.2 in a group 3. Group 1 and 3 SNT showed elevated antibody titers that reached the peak (128) at the 4th-week post-second vaccination and the 3rd-month post-second vaccination respectively, Moreover, antibody titers were maintained within the protective titer zone (≥32) till the 6th month post-second dose/vaccination. Following up, the RVF-SNT antibody titers in the 1st-week post first vaccination showed that it was 3.2 in a group 2 and 4.4 in a group 3. Both groups showed RVF-SNT titers' peak by the 3rd-week post-vaccination by a mean titer of 70.4 in a group 2 and 102.4 in a group 3, then it declined to 3.6 in both groups at the 6th-month post-second dose. Indirect ELISA test results validated and confirmed the SNT results. Simultaneous Vaccination of cattle with BEF and RVF vaccines had no negative effect on the calves' immune response as all vaccinated calves augmented acceptable levels of BEF and RVF specific antibodies. Therefore, calves can be vaccinated simultaneously against BEF and RVF safely with protective titers of antibodies.

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Introduction

Bovine ephemeral fever (BEF) is an acute febrile, arthropod-born viral disease affecting cattle and water buffaloes in tropical and subtropical regions of Africa, Australia, Asia, and the Middle East (Hsieh *et al.*, 2005; Walker, 2005). It is caused by *Ephemerovirus febris* (BEFV) a member of the genus *Ephemerovirus*, subfamily *Alpharhabdovirinae*, family *Rhabdoviridae* (Walker, 2005). The clinical disease is characterized by biphasic fever, ocular and nasal discharge, salivation, recumbence, muscle stiffness, anorexia, lameness, rapid onset of disease, rapid recovery, and remains for 1-3 days (Hsieh *et al.*, 2005; Zheng and Qiu, 2012). BEF gives rise to significant economic losses due to the vast drop in milk production in dairy cattle even after recovery and loss of condition in beef cattle (Davis *et al.*, 1984; Walker, 2005). It was proposed that the virus being introduced to the Middle East through cattle trading from China (Walker and Klement, 2015). There are four types of vaccines used for BEF: Inactivated, live attenuated, subunit G protein-based, and recombinant vaccines (Walker and Klement, 2015; Wallace and Viljoen, 2005). In Egypt, the live attenuated BEF vaccine was produced from Webster, S919 VID-strain through serial passages in susceptible calves till its virulence dropped and adapted to the Vero cell line and considered as the most immunogenic and the safest vaccine for cattle (Daoud *et al.*, 2001a). During an epidemic in Egypt 2000, a binary ethyleneimine (BEI) inactivated BEF vaccine was prepared from local isolates. The vaccine augmented the protective immune response in vaccinated cattle that lasted for six months with two successive doses (2ml, S/C) at four weeks (Daoud *et al.*, 2001b). The live attenuated vaccine of BEF showed higher levels of immunity in vaccinated cattle than the cattle vaccinated with two doses of inactivated vaccines with an interval of 2 weeks. Priming by a dose of a live vaccine, followed by a booster dose of the inactivated vaccine was reported to yield the most optimum immune response in vaccinated cattle (El-Shamy, 2006).

Rift Valley fever (RVF) is a devastating mosquito-borne viral disease characterized by high mortality in young animals, especially in lambs (Flick and Bouloy, 2005; Gerdes, 2004). RVF's most susceptible hosts are sheep, goats, cattle, buffaloes, some wild animals, and mice (Pepin *et al.*, 2010). *Phlebovirus riftense* formerly known as Rift valley fever virus (RVFV) is a single stranded RNA virus that belongs to the genus *Phlebovirus*, family *Phenuviridae,* and order *Bunyavirales* (Maes *et al.*, 2018). The most characteristic clinical sign of RVF infection is the storm of abortion in pregnant ruminants (Daubney *et al.*, 1931). In newborn lambs, the disease is peracute with 95-100% mortality (Olaleye *et al.*, 1996). RVF has a severe economic impact due to the tremendous economic losses in animals and humans affected by outbreaks that happened in Egypt in 1977-1978, 1993, 1994, 1997, and 2003 (Kamal, 2011). In 1977, an explosive outbreak occurred in Egypt, causing infections in about 200,000 people, 600 of them were fatal (Kamal, 2011). Two types of inactivated vaccines are being used in Egypt (1) Formalin-inactivated vaccine with alum adjuvant (Menya/sheep/258) strain that is produced by VACSERA company (Egyptian company for vaccines production), (2) Binary ethylenimine inactivated vaccine with alum adjuvant (ZH501) strain, produced by Veterinary Serum and Vaccines Research Institute (VSVRI), Egypt (Kamal, 2011), and (3) Live Smithburn neurotropic attenuated strain produced by VSVRI, Egypt (Kamal, 2011). The process of vaccine production is intricate, time-consuming, and requires a biosafety level three laboratory (Kortekaas *et al.*, 2011). Because Egypt is considered an RVF-endemic region, it is not safe to use the live-attenuated vaccine (Kamal, 2009). The present study aims to serological detection of both inactivated cell culture BEF vaccine and inactivated RFV vaccine effect on studied Egyptian calves.

Materials and Methods

BEF and RVF vaccines

Locally prepared BEF vaccine strain BEF/Abassia/2000 (Batch No.1/2018) live attenuated that was inactivated at the time of vaccination using its specific diluents (Albehwar *et al.*, 2010) and inactivated RVF vaccine strain Zagazig ZH501 (Batch No. 2/2018) were used to vaccinate the experimental calves as shown in the experimental design. Each vaccine contains not less than 10^7TCID$_{50}$/ml of the virus. These vaccines were supplied by Veterinary Serum and Vaccine Research Institute (VSVRI), Abassia, Cairo, Egypt.

BEF and RVF antigens and cell line

Baby Hamster Kidney 21 (BHK-21) cell culture adapted strain of RVF virus of a titer $10^7\log_{10}$ TCID$_{50}$/ml (Elian *et al.*, 1996) and BEF virus of a titer $10^{7.5}$

log_{10} $TCID_{50}$/ml (Azab *et al.*, 2002) were provided by VSVRI and used in SNT.

Baby Hamster Kidney cells (BHK-21 clone 13) used in SNT was provided from the Animal Virus Institute, Pirbright, UK. They were maintained at RVF and BEF department, VSVRI with Minimum Essential Medium (MEM) with Earl's salts, and 8-10% sterile newborn calf serum.

Animals and vaccination schedule (Experimental design)
Eighteen field crossbreed calves about 6-8 months were housed, in a separate place, in Veterinary Serum and Vaccine Research Institute (VSVRI), Abassia, Cairo, under hygienic measures receiving balanced ration and adequate water, animals were divided into four groups (5 animals/ each for the first three groups and three animals in the 4th group). And all animals were screened using the serum neutralization test (SNT) to be free from RVF and BEF antibodies.

*Group-l: vaccinated with 2ml S/C at the neck side of BEF vaccine, then booster dose after two weeks.
*Group-2: vaccinated with 2ml S/C at the neck side of RVF vaccine, two doses with two weeks intervals.
*Group-3: vaccinated with 2ml S/C at the neck side of BEF vaccine simultaneously by 2ml S/C at the neck side of RVF vaccine, then received a booster doses of both vaccines.
*Group-4: were served as a non-vaccinated control group.

Sampling and serum neutralization test (SNT)
216 blood samples were collected and serum samples were prepared and kept till serological examination according to (Lennette and Schmidt, 1964) Serum samples were collected from all animals before and weekly after vaccination (four times), then monthly up to six months post-vaccination to estimate and follow up the induced antibody levels in experimented animals. Both qualitative and quantitative SNT were done using the microtiter technique according to (Rossiter *et al.*, 1985), and the serum neutralizing antibodies titer was calculated as the reciprocal of the final serum dilution that neutralized and inhibited the cytopathic effect (CPE) of 100-200 $TCID_{50}$ of the virus according to (Singh *et al.*, 1976).

Indirect Enzyme-Linked Immuno-Sorbent Assay (ELISA): To detect the amount of antigen added during the coating procedure which uptake specific antibodies, a modified checkerboard titration was used. According to the combined methods of (Hubschle *et al.*, 1981; Voller *et al.*, 1976).

Statistical analysis
ANOVA test model SAS software (6.12/1996) will be suitable to use. Duncan multiple range test will be good to test the significance among the means (Snedecor and Cochran, 1989).

Results and Discussion

Results of mean BEF serum neutralizing antibodies and ELISA titers in vaccinated calves
There were detectable BEF antibody titers by SNT in the first week post first vaccination as 6 in the group 1 and 5.2 in the group 3. Both groups showed peak SNT titer 128 of BEF virus by the 4th-week post last vaccination dose and the 3rd-month post-second vaccination respectively and remained in this protective titer of antibodies (≥32) until the end of the experiment at the 6th-month post-second dose of vaccination as shown in Figure 1. Indirect ELISA showed that the first dose of the BEF vaccine resulted in detectable antibodies in vaccinated calves by the first week post first vaccination dose as 0.48 log_{10}/ml in the group 1 and 0.71 log_{10}/ml in the group 3. In group 1, it reached the Peak BEF-ELISA titer (2.25 log_{10}/ml) by the third week after the second dose and to 2.55 log_{10}/ml in the group 3 by the 2nd-month post-second vaccination as shown in Figure 2. No antagonizing effect of the BEF vaccine on the calves' immune response to the RVF vaccine was detected. All non-vaccinated calves remained seronegative all over the experimental period.

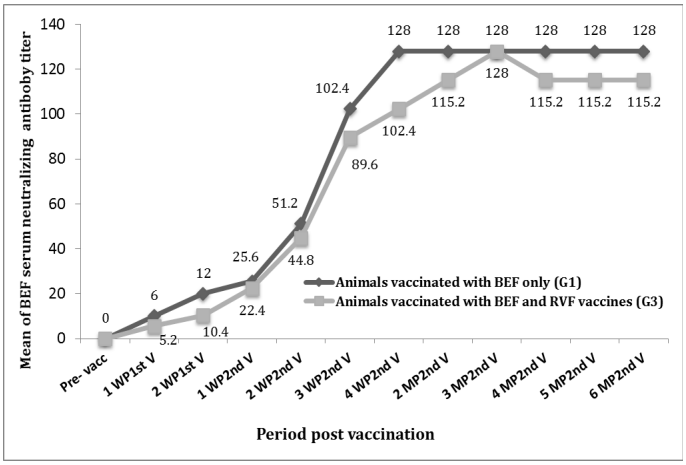

Figure 1: *BEF serum neutralizing antibody titers induced in Calves by single BEF vaccine and simultaneously with RVF vaccine.*

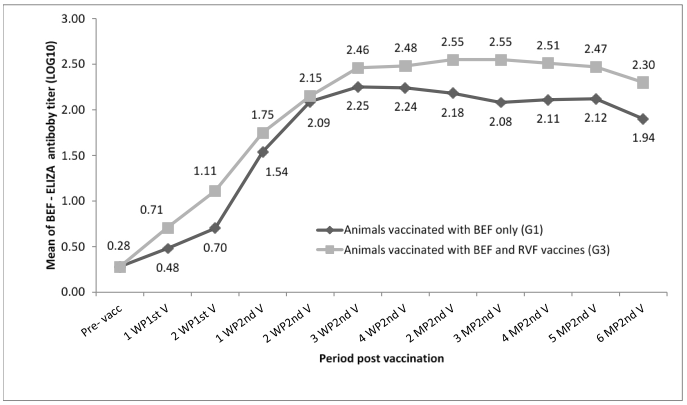

Figure 2: *BEF -ELISA antibody titers induced in calves by single BEF vaccine and simultaneously with RVF vaccine.*

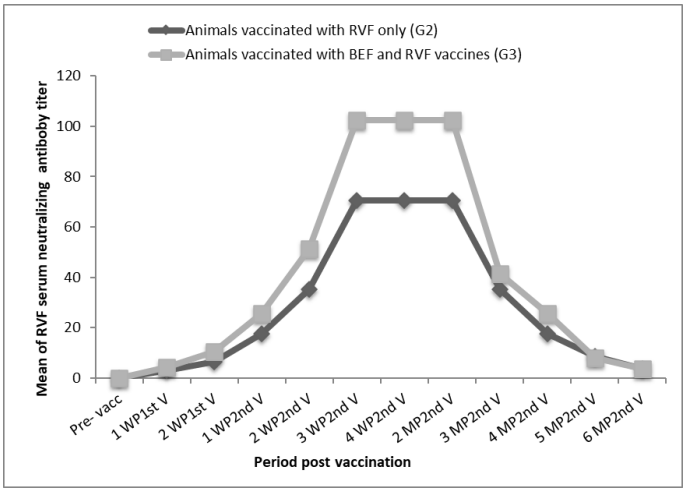

Figure 3: *RVF serum neutralizing antibody titers induced in calves by single RVF vaccine and simultaneously with BEF vaccine.*

Results of mean RVF serum neutralizing antibodies and ELISA titers in vaccinated calves

Following up, RVF antibody titers using the SNT test showed detectable RVF antibodies in the first-week post first vaccination dose as 3.2 in the group 2 and 4.4 in the group 3. Both groups showed peak SNT titer of RVFV by the 3rd-week post last vaccination by a mean titer 70.4 in the group 2 and 102.4 in the group 3, and a declined to 3.6 in the group 2 and group 3 at the end of the experiment as shown in Figure 3. Estimating RVF antibody titers using ELISA showed detectable antibodies in the 1st-week post-first vaccination as 0.71 log_{10}/ml in the group 2 and 0.66 log_{10}/ml in the group 3. RVF titer reached the peak at the 2nd-month post last vaccination by a mean titer 2.46 log_{10}/ml in the group 2 and 2.50 log_{10}/ml in the group 3 at the 4th-week post last vaccination and declined to 1.37 log_{10}/ml in the group 2 and 0.90 log_{10}/ml in the group 3 at the end of the experiment at the 6th-month post-second vaccination by ELISA assay as shown in Figure 4. No antagonizing effect of the RVF vaccine on the calves' immune response to the BEF vaccine was detected. All non-vaccinated calves were tested parallel to the other tested groups and remained seronegative all over the experimental period.

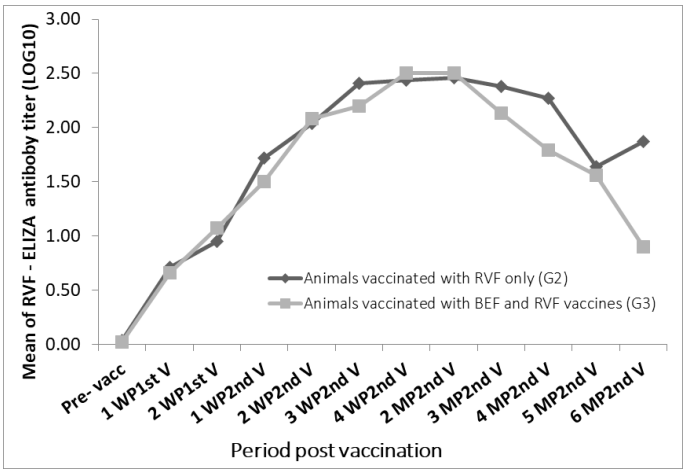

Figure 4: *RVF- ELISA antibody titers induced in calves by single RVF vaccine and simultaneously with BEF vaccine.*

It is well known that vaccination against infectious viral diseases is a cornerstone in protecting susceptible hosts against possible infections. The present study aimed to evaluate the vaccination timing and schedule in protecting cattle against bovine ephemeral fever and Rift Valley Fever viral infections and answer some questions about the possibility of vaccinating cattle with either bovine ephemeral fever or Rift Valley Fever vaccines or with both. The present study showed that calves receiving two doses of the cell culture BEF vaccine that was inactivated at the time of use (inactivated BEF vaccine) with two weeks interval exhibited a detectable level of specific BEF antibodies by the 1st-week post the first vaccination dose. The titers increased gradually after the second dose till the 3rd week after the second vaccination dose and then reached a peak titer of 128 in the 4th week after the last vaccination dose. The antibody titer remained within the protective level up to the 6th-month post-last vaccination dose as shown in Figure 1.

Meanwhile, at the 1st-week post-vaccination, the mean BEF-ELISA antibody titer was 0.48 log_{10}/ml, and it was elevated by the 3rd week after the 2nd vaccination dose and reached a peak of (2.25 log_{10}/ml). After that, it declined to 2.18 log_{10} /ml by the 2nd-month post the last vaccination dose and remain within protective level (≥1.5 log_{10}/ml) till the end of the experiment (1.94 log_{10}/ml), as shown in Figure 2. Similar results were

reported by (Chiu and Lu, 1987; Daoud *et al*., 2001b, 2005; Uren *et al*., 1994; Vanselow *et al*., 1995; Younis *et al*., 2005), they found that vaccination of cattle with two doses of the inactivated BEF vaccine was capable of producing high immunity against BEF virus infection.

Also, utilization of live attenuated BEFV that was inactivated at the time of administration induced high levels of specific BEF neutralizing antibodies due to the saponin content of the diluents, which acts as a virus inactivator and immune stimulant (Albehwar *et al*., 2010; Ellis *et al*., 2005). Saponins were found to possess antimicrobial characters, protecting the host against viruses, bacteria, and fungi. At the same time, they improve the immune system's function by stimulating the production of T-cells. They also can act as antioxidants and scavenge oxidative stress (Cibulski *et al*., 2018; Francis *et al*., 2002).

Calves vaccinated with two doses of the inactivated RVF vaccine elicited RVFV neutralizing antibodies as demonstrated by SNT with a mean titer 3.2 by the 1st week after the first vaccination, reaching a peak titer of 70.4 by the 3rd week after administration of the second dose. This titer began to decline by the 3rd month after the second dose (35.2), recording its lowest value (3.6) by the 6th month after the last vaccination as in Figure 3. These results agreed with those obtained by (El-Nimr, 1980; Eman, 1995; Gihan, 1990; Gihan and Elian, 1997) that RVF antibodies peak level (128) produced at the 2nd-month post administration of the second dose of the inactivated vaccine. Also, (Abd El-Samea *et al*., 1994) reported that sheep vaccinated with the inactivated RVF vaccine had a protective titer of antibodies up to 4 months. More recent studies confirmed our present results where the inactivated RVF vaccine was found to enhance the peak titer of antibodies (128 by SNT) in vaccinated animals by the 2nd month after vaccination, then it declined to 32 by the 7th month (El-Bagoury *et al*., 2017). ELISA results showed that vaccinated animals with RVF vaccine alone provided detectable titers of specific RVF antibodies by the 1st-week post first vaccination with a mean titer 0.71 $\log_{10}$/ml reaching a mean peak titer of (2.46 $\log_{10}$/ml) by the 2nd-month post the last vaccination dose. This titer was then declined to 2.38 $\log_{10}$/ml by the 3rd month after the second vaccination reaching the lowest value 1.37 $\log_{10}$/ml by the 6th-month post the last vaccination, as shown in Figure 4. ELISA results confirmed the results of SNT, as it was considered an accurate and sensitive test to evaluate the immune status of RVF in animals as stated by (Botros *et al*., 2006; Paweska *et al*., 2003a, b), they obtained similar results after vaccination of sheep or cattle with the inactivated RVF vaccine. SNT and ELISA results showed that there is no adverse effect of the BEF vaccine on the calves' immune response to the RVF vaccine, where animals either vaccinated only with the RVF vaccine or simultaneously with BEF exhibited high levels of specific BEF antibodies as demonstrated in Tables 1 and 2.

Table 1: *Results of mean BEF serum neutralizing antibodies and ELISA titers in vaccinated calves.*

Period post vaccination	BEF serum neutralizing antibody titer*			Log_{10} BEF-ELISA antibody titer		
	Group-1	Group-3	Group-4	Group-1	Group-3	Group-4
Pre-V**	0	0	0	0.28	0.28	0.03
1WP1st V***	6	5.2	0	0.48	0.71	0.03
2WP1st V	12	10.4	0	0.70	1.11	0.03
Administration of the 2nd dose						
1WP2nd V****	25.6	22.4	0	1.54	1.75	0.02
2WP2nd V	51.2	44.8	0	2.09	2.15	0.03
3WP2nd V	102.4	89.6	0	2.25	2.46	0.03
4WP2nd V	128	102.4	0	2.24	2.48	0.03
2MP2ndV*****	128	115.2	0	2.18	2.55	0.02
3MP2ndV	128	128	0	2.08	2.55	0.03
4MP2ndV	128	115.2	0	2.11	2.51	0.02
5MP2ndV	128	115.2	0	2.12	2.47	0.03
6MP2ndV	128	115.2	0	1.94	2.30	0.02

**Serum neutralizing antibody titer= the reciprocal of the final serum dilution which neutralized 100 $TCID_{50}$ of BEF virus. **Pre-V= pre-vaccination. ***WP1st V= week post first vaccination. ****WP2nd V= week post-second vaccination. *****MP2nd = month post-second vaccination. Group-1: Vaccinated with BEF vaccine alone. Group-3: vaccinated simultaneously with BEF and RVF vaccines. Group-4: kept without vaccination as a test control.*

Table 2: *Results of mean RVF serum neutralizing antibodies and ELISA titers in vaccinated calves.*

Period post vaccination	RVF serum neutralizing antibody titer*			Log_{10} RVF-ELISA antibody titer		
	Group-2	Group-3	Group-4	Group-2	Group-3	Group-4
Pre-V**	0	0	0	0.03	0.02	0.03
1WP1st V***	3.2	4.4	0	0.71	0.66	0.03
2WP1st V	6.4	10.4	0	0.95	1.07	0.02
Administration of the 2nd dose						
1WP2nd V*** *	17.6	25.6	0	1.72	1.50	0.03
2WP2nd V	35.2	51.2	0	2.04	2.08	0.03
3WP2nd V	70.4	102.4	0	2.41	2.22	0.05
4WP2nd V	70.4	102.4	0	2.44	2.50	0.03
2MP2ndV***, **	70.4	102.4	0	2.46	2.50	0.03
3MP2ndV	35.2	41.6	0	2.38	2.19	0.02
4MP2ndV	17.6	25.6	0	2.27	1.79	0.02
5MP2ndV	8.8	8	0	1.92	1.56	0.03
6MP2ndV	3.6	3.6	0	1.37	0.90	0.02

**Serum neutralizing antibody titer= the reciprocal of the final serum dilution which neutralized 100 $TCID_{50}$ of BEF virus. **Pre-V= pre-vaccination. ***WP1st V= week post first vaccination. ****WP2nd V= week post-second vaccination. *****MP2nd = month post-second vaccination. Group-2: Vaccinated with RVF vaccine alone. Group-3: vaccinated simultaneously with BEF and RVF vaccines. Group-4: kept without vaccination as a test control.*

Although there were very little data that discussed the relationship between the effect of BEF and RVF vaccines on the immune response of vaccinated animals; the cumulative Tables 1, 2 revealed that calves vaccinated simultaneously with two doses of both BEF and RVF vaccines with two weeks interval showed specific BEF serum neutralizing antibodies by a mean titer of 5.2 by the 1st-week post the first dose then increased gradually after the second dose in the 3rd month by a peak titer of 128 then slightly declined to be 115.2 by the 4th-month post the second vaccination but still within such high protective level up to the 6th month later as shown in Figure 1. The BEF-ELISA titer by the 1st-week posts the first vaccination was 0.71 log_{10}/ml showing its peak (2.55 log_{10}/ml) by the 2nd month after the 2nd vaccination then declined gradually to be 2.30 log_{10}/ml by the 6th-month post last vaccination, as shown in Figure 2. In calves vaccinated simultaneously with BEF and RVF vaccines, calves exhibited RVF serum neutralizing antibody titers by the 2nd-week post the 1st vaccination by a mean titer 10.4 which was higher than that induced in only RVF vaccinated animals by a mean titer of 6.4. Such titers reached their peak (102.4 and 70.4) by the 3rd week post-2nd vaccination dose, respectively, as shown in Figure 3. Comparable results were obtained by ELISA showing titers of 1.07 log_{10}/ml and 0.95 log_{10}/ml on the 2nd-week post the 1st vaccination dose and reached to the peak by a mean titer 2.50 log_{10}/ml at 4th-week post-second vaccination and 2.46 log_{10}/ml by the 2nd-month post second vaccination in simultaneous and single vaccination respectively as showed in Figure 4. Such observation could be attributed to saponin's presence in the diluents of the BEF vaccine that can act as an immune stimulant (Cibulski *et al.*, 2018; Francis *et al.*, 2002) and virus inactivate (Ellis *et al.*, 2005), On the other hand, no adverse effect of inactivated RVF vaccine on animal response to other vaccines was noted. Similar results with vaccination of sheep or cattle simultaneously with both BEF and RVF vaccines were found (Abd El-Samea *et al.*, 1994; El-Shamy, 2006; Khodeir *et al.*, 1998).

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Novelty Statement

Our results show that Indirect ELISA test results validated and confirmed the SNT results also; simultaneous vaccination of cattle with BEF and RVF vaccines had no negative effect on the calves' immune response as all vaccinated calves augmented acceptable

levels of BEF and RVF specific antibodies. Therefore, calves can be vaccinated simultaneously against BEF and RVF safely with protective titres of antibodies.

Author's Contribution

FM, SAA: Organized the whole process and drafted the manuscript. FM, E-TMMA: Designed the work. SAA, FM: Performed the work. FM, SAA and EKE: Performed the data analysis. FM and SAA: Wrote the work. All authors read and approved the final manuscript.

Ethical approval

All animals used in this study were handled and cared for according to the animal care and use criteria approved by the animal care and use committee of faculty of veterinary medicine, Suez Canal University, Ismailia, Egypt. Was approved by committee with number (SCU 2023093).

Conflict of interest

The authors have declared no conflict of interest.

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