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Immunogenicity of a freeze-dried combined vaccine against Rift Valley fever and bovine ephemeral fever in cattle

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Abstract

Background: The target of vaccination is to encourage a strong, covering and long-lasting immune response against antigens. For achieving these objectives; effective adjuvant and new vaccine strategies are demanded to make the vaccine sufficiently immunogenic to instigate a powerful immune response

Aim: This study was completed for elaboration and evaluation of freeze-dried combined vaccine against both Rift Valley fever (RVF) and bovine ephemeral fever (BEF) viruses using different stabilizers.

Methods: Three formulae were prepared from such vaccine including: formula (1): stabilized with a mixture of 5% Lactalbumin Hydrolysate and 2.5% sucrose, formula (2): stabilized with a mixture of 50% the previous stabilizer and 50% of 1% Carbopol and formula (3): stabilized with 1% Carbopol solution. Samples of the three vaccine formulae were reconstituted on the time of experimental animal vaccination using saponin diluent which acts as an adjuvant for both RVFv and BEFv and as an inactivator BEF virus. The ratio between both viruses in all vaccine formulae was 1:1.

Results: All vaccine batches were proved to be free of any foreign contaminants and unharmed for experimentally vaccinated animals. Each of the three groups of calves was vaccinated S/C with 2 ml of a reconstituted vaccine formula and their immune response was evaluated using serum neutralization test. The gained results revealed that the prepared combined freeze-dried vaccine with Carbopol elicited a better humoral immune response than the other two vaccine formulae.

Conclusion: It could be recommended to use Carbopol as a stabilizer for the preparation of the aimed vaccine.

Keywords: Rift Valley fever, Bovine ephemeral fever, Neutralization test, Cytopathic effect.

Introduction

Rift Valley fever (RVF) is a zoonosis viral disease that mainly affects animals and human beings. It was initially specified in 1931 in a sheep epidemic in the Rift Valley in Kenya. Even after, RVF outbreaks were reported in sub-Saharan Africa and Egypt. Cases were assured in Saudi Arabia and Yemen in 2000, and alarmist that it could expand to other areas of Asia and Europe. Mainly infections in humans, result from handling the blood and organs related to the infected animals (WHO, 2020). Mainly, the disease affected small and large ruminants, camels and humans causing general weakness, fever, salivation, fetid diarrhea, and reduction in milk yield with a storm of abortions in pregnant animals (FAO, 2003). The outbreak of RVF

was associated with heavy rainfalls due to mosquito spreading (Nguku *et al.*, 2006). The preferable device for protecting animal inhabitation and indirectly humane beings is vector hold and vaccination by using secure and potent vaccines (Abdel Ghaffar *et al.*, 1979). RVF vaccines are two types, live attenuated Smithburn vaccine and adjuvanted inactivated vaccine (Faburay *et al.*, 2017). The limitation of applying live RVF vaccine is due to teratogenic or abortogenic action (Hubbard *et al.*, 1991). So, the inactivated RVF vaccine is mainly used in Egypt with two doses for inducing high protection levels of antibody titer and long period (OIE, 2016).

On the other side, bovine ephemeral fever (BEF), or 3-day sickness (Akakpo, 2015) is a non-contagious epizootic arthropod-borne Rhabdovirus disease (mostly

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likely mosquitoes) mainly affects cattle and water buffalo, coming across tropical and subtropical zones of Asia, Australia, and Africa. The disease is marked by a short fever, shivering, lameness, and muscular stiffness, followed by inability to stand, reluctance to move, anorexia, ocular and nasal discharge, and excessive salivation. The disease may cause serious economic losses through deaths, decreased milk production mortality is usually low when diseased animals receive compatible medical attendance (George *et al.*, 1984; Uren *et al.*, 1992). The disease's importance is related to economic losses, as it causes a marked drop in milk output with deprivation in beef cattle conditions (Walker, 2005; Akakpo, 2015). Vaccination of calves with steady boosts and quality vaccines usually induces efficient protection (Nandi and Negi, 1999). The current used vaccine against RVFv is the aluminum hydroxide gel inactivated RVF vaccine which can be administered to all animals of all ages, and it requires repeated doses to induce and maintain protective immunity since an initial dose may only give protection for 7 months (El-Bagoury *et al.*, 2013), while vaccination against BEFv is applied using SERVAC BEF attenuated vaccine which induce duration of immunity about 1 year (Elgendy *et al.*, 2021)

The current trend is using various microorganisms in a combined vaccine is highly sustain the protection against the diseases in livestock aside from conserving costs, efforts, and time during vaccination

The target of immunization by vaccines is to encourage a potent, covering and prolonged immune response against the antigen. To achieve these objectives; effective adjuvant and new vaccine production are demanded to make the vaccine sufficiently immunogenic to instigate a powerful immune restraint (Fearon, 1997; Bomford, 1998).

Carbopol is a synthetic polymer that has many implementations in pharmaceuticals. The aqueous Carbopol gel is compatible with many ingredients, thermostable, and flow easily during different routes of application (Islam *et al.*, 2004). Carbopol has various advantages like high safety, nontoxic, and suspending agent (Ahuja *et al.*, 1997). Carbopol can promote and animate cellular and humoral immunity in mammals (Gartlan *et al.*, 2016). The feature of using aquatic Carbopol gel is its easy-flowing manner, its affinity with many active ingredients; and its thermal stability (Zhang *et al.*, 2018).

Saponins, extracted from *Quillaia Saponaria* Molina, it is widely used as adjuvants for several years and has been used in sundry veterinary vaccines. The adjuvants have the capability to modulate the cell-mediated immune system and promote antibody fabrication (Oda *et al.*, 2000). It motivates a potent production of T-dependent as well as T-independent antigens, and cytokines such as interleukins and interferon that might intercede with their immune stimulant effects (Jie *et al.*, 1984; Kensil, 1996).

So, the definitive goal of this work is an elaboration of a combined inactivated RVF and BEF vaccine in a lyophilized form using high-quality stabilizer to increase its stability saving efforts and time consumed during vaccine production.

Materials and Methods

Experimental animals

Swiss albino baby mice

Ten baby mice (3-4 days old) were used for safety tests to determine the complete inactivation of RVF virus.

Calves

Twenty native breeds, non-vaccinated healthy calves, about 12 to 18 months old were tested and confirmed to be free from RVF and BEF antibodies as screened by serum neutralization test (SNT). They were classified into four groups, each one of the first three groups contains 6 animals vaccinated with one dose of one of the prepared combined vaccine formulae. A booster dose of vaccination was administered to only three calves from each group after one month. The fourth group contains two calves kept as unvaccinated control group. All calves' groups were kept under strict hygienic conditions in insect proof stables receiving balanced ration and adequate water with daily clinical examinations. In addition, another 10 calves were used to test the safety of the prepared vaccine formulae.

Viruses

Virulent RVFV (ZH 501) was supplied by the Department of Rift Valley Fever Vaccine Research; Veterinary Serum and Vaccine Research Institute (VSVRI), Abasia Cairo, cultivated on BHK cells with final concentration 10^8 TCID₅₀ / ml and used for the of lyophilized combined RVF and BEF vaccine.

BEF virus strain

BEFV/abasia/2000 (Soliman *et al.*, 2001) was supplied by the Department of Pet Animal Vaccine Research, VSVRI; cultivated on BHK cells with final concentration 10^8 TCID₅₀ / ml and employed for vaccine elaboration and SNT.

Tissue culture

Baby hamster kidney cell line (BHK21) supplied by VSVRI were passaged and preserved according to Macpherson and Stocker (1962) and used for virus propagation, titration, vaccine elaboration and SNT.

Chemicals

Binary ethylenimine

0.1 M Binary ethyleneimine (BEI) stock solution prepared from 2 Bromo ethylamine hydrobromide (Aldrich Chemical Co., LTD) and 0.2 N NaOH, according to Bahnemann (1990) and Mark (2004) to be used for RVF virus inactivation process.

Carbopol

It was supplied by Lubrizol as powder and dissolved in hot water to make 1% aqueous stock solutions (United States Pharmacopeial Convention Inc. 2022). The prepared solutions were exposed to heat sterilization by autoclaving at 121°C for 20 minutes, after that it was stored at 4°C until next use.

Saponin

Saponin was supplied by Sigma-Aldrich Labo Chemikalien Gm6H; Germany (cat. No.16109; lot.71500). It was prepared as watery solution in a concentration of 0.5 mg /ml in phosphate-buffered saline as described by Amoros *et al.* (1987) while was used as a diluent and to inactivate the live attenuated BEF virus vaccine just before vaccination.

Stabilizers

A stabilizer formed from 5% lactalbumin hydrolysate (Sigma-Aldrich GmbH) with 2.5% sucrose was prepared according to Riyesh *et al.* (2011). Three stabilizers were used for elaboration of freeze-dried formulae of the RVF and BEF combined vaccine as follow:

Stabilizer-1 composed of 5% Lactalbumin Hydrolysate and 2.5% sucrose

Stabilizer-2 composed of a mixture of stabilizer-1 and 50% of 1%carbopol

Stabilizer-3 composed of 1% Carbopol solution

Virus titration

Both of RVF and BEF viruses were titrated in BHK21 cell culture (Fig. 1a) using the microtiter method according to Rossiter *et al.* (1985) and the virus titer was calculated as \log_{10} TCID₅₀/ml according to Reed and Muench (1938).

Virus inactivation

RVF virus was inactivated by using BEI according to Bahnemann (1990) and Mark (2004) while BEF was inactivated with saponin at the time of vaccination according to Albehwar *et al.* (2010).

Checking of complete RVF virus inactivation (Safety test)

Inactivated RVF virus samples were tested for the existence of vigorous virus in tissue culture according to (OIE 2012) by inoculating BHK confluent monolayer with the inactivated virus to be detected with daily microscopic examination and in baby mice according to El Nimr (1980) and Eman (1995) by inoculating baby mice (3-5 days old) with 0.03 ml of inactivated virus then mice were kept for 10 days with daily examination. Mice dying within the first 24 hours were discarded. The inactivated virus was approved as safe if there is no cytopathic effect (CPE) in tissue culture and if all inoculated mice survived for 10 days.

Preparation of combined freeze-dried RVF and BEF vaccine

After propagation and titration of both viruses, the virus titer of used RVF virus was 10^8 and BEF virus titer was 10^8 TCID₅₀/ml. Live BEF virus was mixed altogether with inactivated RVF in equal volumes to prepare three formulae of the combined lyophilized vaccine:

Formula-1 was prepared by mixing the virus mixture with an equal volume of stabilizer-1 (5% lactalbumin hydrolysate and 2.5% sucrose).

Formula-2 was prepared by mixing the virus mixture with an equal volume of stabilizer-2 (50% {5%Lactalbumin Hydrolysate with 2.5% sucrose} and 50% of 1% Carbopol).

Formual-3 was prepared by mixing the virus mixture with an equal volume of 1% Carbopol. Each vaccine formula was dispensed in neutral glass vials (2.5 ml/vial) and exposed to the freeze drying process.

Each vial of vaccine formula was reconstituted in 10 ml Saponin (0.5 mg/ml) according to Amoros *et al.* (1987) as adjuvant just before inoculation where each vaccinal dose contains 10^6 TCID₅₀/ml of RVF and 10^6 TCID₅₀/ml of BEF

Quality control tests

Sterility test

Randomly picked up samples from the virus fluids and the final products were checked for their liberty of odd contaminants (aerobic and anaerobic bacteria, fungi, and mycoplasma) on Nutrient agar medium; Thioglycolate medium (Oxford, England); Sabouraud dextrose agar medium and PPLO medium according to the recommendation of the Code of Federal Regulations (2005) and OIE (2015).

Safety test

Vaccine safety test was carried out to test the safety of both RVF and BEF in calves (the target host). Each of the three calves was inoculated subcutaneously with 10 ml from each formula of the prepared combined lyophilized vaccine keeping a fourth calf without vaccination as control. All calves were observed daily for 2 weeks for detection of any post-vaccinal reactions.

Potency test

Each formula of the ready lyophilized combined RVF and BEF vaccine was employed to vaccinate a calves' group followed by monitoring of their immune response to RVF and BEF viruses using SNT microtiter

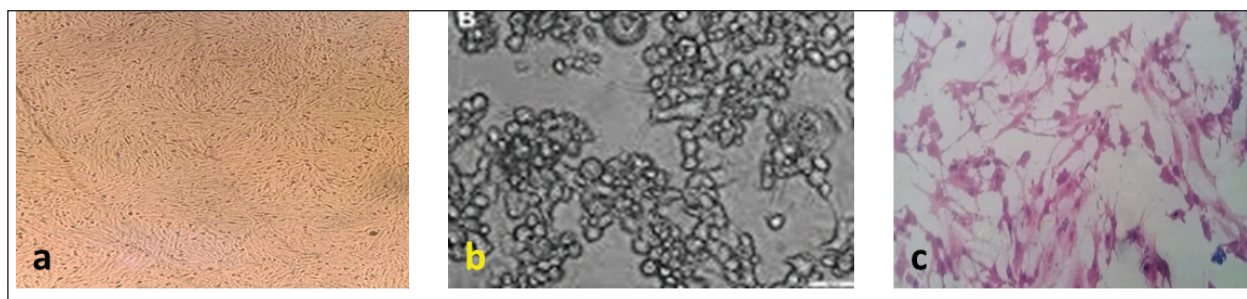


Fig. 1. (a) Uninfected BHK control cells. (b) CPE of RVFv in BHK. (c) CPE of BEFv in BHK.

technique as described by Rossiter *et al.* (1985) while the titer of the obtained antibodies was elaborated according to Reed and Muench (1938).

Schedule of calves' vaccination

Each vaccine formula was reconstituted in 10 ml Saponin (0.5 mg/ml) as an adjuvant and inactivator to BEF virus at the time of inoculation as follows using vaccination dose of 2 ml/calf injected S/C in the neck side:

- Group (1): vaccinated with vaccine formula-1
- Group (2): vaccinated with vaccine formula-2
- Group (3): vaccinated with vaccine formula-3
- Group (4): was kept as unvaccinated control.

Three calves from each of the first three groups received a booster dose at the first month post vaccination (MPV) with the corresponding vaccine formula

Samples

Blood samples were collected from all calves' groups in sterilized, dry and clean screw capped bottles incubated at 37°C for 30 minutes then Kept overnight in a refrigerator. The formed serum was separated and centrifuged at 3,000 rpm for 10 minutes and kept at -20°C till subjected to serological test. Such samples were collected before vaccination then after 2 weeks before booster and then on monthly intervals for evaluation of the induced neutralizing BEF and RVF antibodies.

Ethical approval

The utilize of animals and their care were donated by the medical and Veterinary Research Ethics Board at the National Research Center in Egypt.

Results

Proliferation and titration of RVF virus (ZH501) in tissue culture

Under Biosafety measures; the virus ($10^{7.5}$ TCID₅₀ / ml) was generated and proliferated in BHK cells for three successive times to excess the virus output and the virus titer reached after the three successive passages to

10^8 TCID₅₀ / ml. A notable CPE of RVF virus showed as cell rounding and accumulation in clusters like grapes like aggregation as shown in Figure 1b and Table 1.

Proliferation and titration of BEF virus in tissue culture

The virus ($10^{6.8}$ TCID₅₀ / ml) was generated and proliferated in BHK cells for three consecutive times to rise the virus product till reached 10^8 TCID₅₀ / ml. A notable CPE of BEF virus in BHK cells appeared as cell degeneration that end with the splitting of cells from the culture surface leaving hollow spaces. These stages of cellular changes take place within the initial 24 hours post infection and end usually within 72 hours post infection as shown in Figure 1c and Table 1.

Determination of complete RVF virus inactivation

Inoculation of baby mice and in tissue culture with the inactivated RVF virus revealed its safety showing no mortalities or any abnormal signs of sickness in inoculated mice through 10 days post inoculation observation period and no CPE was observed in inoculated BHK cells,

Quality control testing of the three prepared combines inactivated RVF and BEF vaccine formulae, revealed that all of them were free from foreign contaminants; safe in inoculated calves showing no any abnormal local or systemic clinical signs, and efficient inducing good levels of particular RVF and BEF antibodies in vaccinated calves as shown in Table 2.

Monitoring of RVF neutralizing antibody levels in vaccinated calves using SNT showed that the peak NI was recorded on the second month (3.2) in Boostered calves in group-1 (vaccinated with formula-1) and on the third month in non-Boostered calves in the same group. Boostered calves in group-2 (vaccinated with formula-2) showed their peak of NI (3.6) on the third MPV while non-Boostered calves showed their peak NI (3.2) by the fourth MPV. The third calves 'group had a peak of NI (3.88) in Boostered calves by the fourth (MPV) and 3.6 by the fifth MPV in non-Boostered calve while the non-vaccinated group showed non-protective. The recorded RVF neutralizing antibodies remained within the protective levels up to 9 and 10 MPV in Boostered calves in group-1 and other groups respectively as tabulated in Table 3.

SNT achieved on serum samples gained from various vaccinated calves 'groups showed that BEF neutralizing antibody levels in vaccinated calves showed that their peak of NI on the third MPV (2.5 and 1.7) in Boostered

Table 1. Titers of RVF virus (ZH501) and BEF virus in BHK cells.

Titrated virus	Initial viral titer	Virus titer (log10 TCID ₅₀ /ml)		
		First passage	Second passage	Third passage
RVFV	10 ⁷	10 ^{7.5}	10 ^{7.8}	10 ⁸
BEFV	10 ⁵	10 ^{6.8}	10 ^{7.2}	10 ⁸

Table 2. Quality control measures of Freeze-dried combined RVF and BEF vaccine.

Tested formula	Quality measures		
	Sterility test	Safety test	Potency test
Formula (1)	Disposed of mycoplasma, aerobic and anaerobic bacteria and fungi.	All formulae were safe inducing no any post vaccinal reactions	All vaccine formulae were potent as evaluated by SNT.
Formula (2)			
Formula (3)			

Table 3. Mean RVF antibody neutralizing indices in calves vaccinated with the prepared formulae of the freeze dried combined RVF and BEF vaccine.

Time of Sampling	Mean RVF antibody neutralizing indices in vaccinated calves 'groups						
	Group 1		Group 2		Group 3		Group 4
	Boostered	Non Boostered	Boostered	Non Boostered	Boostered	Non Boostered	Non vaccinated
Pre	0.34	0.30	0.38	0.36	0.28	0.38	0.30
2 WPV*	1.2	1.4	2.2	2	2.8	2.4	0.44
1st MPV**	2.6	2.4	2.8	2.8	3	2.8	0.34
2nd MPV	3.2	2.8	3.4	3	3.6	3	0.36
3rd MPV	3	3	3.6	3	3.8	3.2	0.52
4th MPV	2.8	2.6	3.4	3.2	3.88	3.4	0.6
5th MPV	2.6	2.4	3	2.88	3.6	3.6	0.46
6th MPV	2.4	1.8	2.8	2.48	3.4	3	0.22
7th MPV	2.2	1.4	2.6	2.2	3	2.4	0.22
8th MPV	2	1.2	2.2	2	2.8	2	0.24
9th MPV	1.8	1	2	1.8	2.4	1.8	0.46
10th MPV	1.2	0.8	1.8	1.6	2	1.8	

(*WPV): week post vaccination; (**MPV): month post vaccination; (Group 1): vaccinated with formula (1); (Group 2): vaccinated with formula (2); (Group 3): vaccinated with formula (3); (Group 4): non vaccinated control.

Table 4. Mean BEF antibody neutralizing indices in calves vaccinated with different formula of the prepared freeze-dried combined RVF and BEF vaccine.

Time of Sampling	Mean BEF antibody neutralizing indices in vaccinated calves' groups						
	Group 1		Group 2		Group 3		Group 4
	Boostered	Non Boostered	Boostered	Non Boostered	Boostered	Non Boostered	Non vaccinated
Pre	0.34	0.30	0.36	0.38	0.28	0.26	0.30
2 WPV*	1.1	0.9	2.15	1.2	1.3	1.2	0.44
1st MPV**	1.3	1.2	1.6	1.3	1.5	1.5	0.34
2nd MPV	2.3	1.7	2.8	2.1	2.7	2.2	0.36
3rd MPV	2.5	1.7	3.0	2.3	2.9	2.5	0.52
4th MPV	2.4	1.5	3.1	2.4	3.0	2.5	0.6
5th MPV	2.4	1.2	3.1	2.5	3.1	2.6	0.46
6th MPV	2.1	1.0	3.0	2.5	2.9	2.4	0.22
7th MPV	1.8	0.9	2.8	2.	2.7	2.4	0.22
8th MPV	1.8	0.9	2.6	2.4	2.5	2.1	0.24
9th MPV	1.6	0.3	2.4	2.1	2.2	2	0.46
10th MPV	1.5	0.3	2.1	1.7	1.9	1.8	

(*WPV): week post vaccination; (**MPV): month post vaccination; (Group 1): vaccinated with formula (1); (Group 2): vaccinated with formula (2); (Group 3): vaccinated with formula (3); (Group 4): non vaccinated control.

and non-Boostered calves respectively in group-1(vaccinated with formula-1). Boostered calves in group-2 (vaccinated with formula-2) showed their peak of NI (3.1) on the fourth MPV while non-Boostered

calves showed their peak NI (2.5) by the fifth MPV. The third calves' group had a peak of NI (3.1 and 2.6) in Boostered and non-Boostered calves respectively by the fifth MPV as shown in Table 4. Also, this

table indicates that BEF neutralizing antibodies still remained within their protective levels up to the 9th and 10th MPV in Boostered calves in group-1 and other vaccinated groups respectively.

Discussion

Vaccines fundamentally improve the immune system's capability to react rapidly against microorganisms after a second defiance and have been qualified as 'weapons of mass protection' (Cohen and Marshall, 2001; Curtiss, 2002). The target of vaccination is to invigorate a potent, protective and prolonged immune response to the administered antigen. To perform these goals, efficient adjuvant and vaccine strategies are needed to make the vaccine to an adequate degree immunogenic, for instigating a strong immune response (Fearon, 1997; Bomford, 1998).

This study includes elaboration and evaluation of three different formulae of a freeze – dried combined vaccine against RVF and BEF.

Concerning RVF and BEF virus titrations through the infectivity method in BHK cell line; Table 1 showed that the titer of RVF virus was 10^8 TCID₅₀/ml with CPE represented by cell rounded and aggregated in clusters (Billecocq *et al.*, 1996), while the titer of BEF was 10^8 TCID₅₀/ml with CPE as degeneration and detachment from the culture surfaces (Azab *et al.*, 2000).

Determination of virus inactivation fulfillment in mice and tissue culture (Table 2), confirmed satisfactory results for 14 days post-inoculation with no clinical abnormalities or deaths in agreement with the recommendation of Code of Federal Regulations (2005). The intended lyophilized combined vaccine was confirmed to be sterile and free of mycoplasma, aerobic, anaerobic bacteria, and fungal contamination and safe for inoculated calves showing no elevation in body temperature which stayed within the physical levels for successive 14 days post-vaccination without clinical abnormalities or deaths (Table 3) agreed with Wassel *et al.* (1996) and Code of Federal Regulations (2005) who stated that the eventual product should be free of foreign contaminants and safe in animals.

The serum neutralization test as shown in Table 3, revealed that the RVF serum neutralizing antibody titers reached their protective NI (1.5) by the second WPV in group 2 and 3 and after 1 month in the first group showing an increasing till reaching its peak by the second MPV (3.2) when received the second doses, while in the non-Boostered group the level of antibodies reached its peak at the third MPV then decline to non-protective level after the ninth MPV (1.2). Booster animals in groups (2) and (3), exhibited the peak of their antibody titer by the third to fourth MPV and stayed within the covering grade till the end time of the experiment in agreement with Atwa *et al.* (2020) with little difference between the two groups, while the non-booster animals in group 2 and 3 had levels of RVF antibodies less than those in booster animals

but higher than those in the sera of the vaccinated calves in the first group which although received two doses, and stayed also within the protective level till the ending of the experiment. These results agree with Atwa *et al.* (2020) who reported that the immune response in sheep vaccinated with lyophilized RVF vaccine using lactalbumin and sucrose as stabilizers and diluted with saponin, stayed within the protective level till the end of ninth MPV with two doses and they also mentioned that these results related to the using of saponin as adjuvant. Also, all the previous results come coincidence with Oda *et al.* (2000), who reported that Saponin based adjuvants can modify the cell mediated immune response as well as enhancement of antibody fabrication and have the merit that only a minimal dose is needed for adjuvant activity.

BEF serum neutralizing antibody NI showed detectable levels (1.2–1.3) as mentioned by Kensil (1996) started from the second WPV in group 2 and 3 and reached the protective level after 1 month in the first group which showed an increasing in their level till reach the peak (2.5) 3 MPV receiving 2 doses. The non-booster group exhibited the peak of BEF-NI at the second MPV then declined by the sixth MPV (1.0). In groups 2 and 3, the peak of antibody NI was recorded by the fourth to the fifth MPV and still protective till the end time of the experiment (10 MPV) with a little difference between the two groups receiving two doses of the vaccine with 1 month interval. The non-booster animals in group 2 and 3 had level of antibodies less than that in booster animals but still higher than that in the sera of calves in the first group which although received 2 doses. These findings agree with Gartlan *et al.* (2016) who mentioned that Carbopol (carbomer) enhances and activates cellular and humoral immunity in mammals and the same conclusions were reported by Mair *et al.* (2015) and Aly *et al.* (2020). In addition, Albehwar *et al.* (2010) stated that the use of a live BEF vaccine which was inactivated at the time of administration induced high levels of specific BEF neutralizing antibodies where the diluent of such vaccine contains saponin which acts as a virus in activator and immune stimulant.

Conclusion

The present obtained results showed the possibility of elaboration of the combined RVF and BEF lyophilized vaccine with Carbopol for eliciting a good level of protective immunity against the two diseases in cattle with accepted duration of immunity.

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Authors contributions

Naglaa Ebrahim Aly: Research idea; Preparation of lyophilized vaccine; Collection of blood and serum

samples in order to track the level of blood immunity in calves after vaccination; Participating in writing the research. Mohamed Hassan Atwa: Conducting a test for the efficiency and effectiveness of the prepared vaccine; Participating in writing and publishing the research. Amany Mohamed Abbas: Participating in preparation of lyophilized vaccine; Participating in publishing the research. Diana Mohamed Abulmagd: Conducting a test for the efficiency and effectiveness of the prepared vaccine; Following up the induced immunoglobulins throughout the experiment; Evaluation and analysis the results; Writing the research; Participating publishing the research. Zeinab Taha Salem: Participating in preparation of lyophilized vaccine; Evaluation and analysis the results; Participating in publishing the research. Taradi Abdelfattah Sayed: Evaluation and analysis the results; Participating in writing the research.

Conflict of interest

The authors declare that they have no competing interests.

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Availability of data

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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