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TRIAL FOR PREPARATION OF COMBINED INACTIVATED RVF AND SHIPPING FEVER VACCINE IN SHEEP

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ABSTRACT

Preparation and evaluation of a combined inactivated Rift Valley fever (RVF) and Shipping fever (SF) vaccine adjuvanted with Montanide™ ISA 206 was the aim. Single RVF vaccine and single SF vaccine were prepared with the combined one. These vaccines were sterile; safe and evaluated in 4 groups of sheep. The groups were vaccinated as follow; the first with RVF, the second with SF, the third with the combined vaccine, but the fourth was kept as a negative control. Each vaccinated group received two doses of its vaccine (1ml/dose); 1 month apart. The vaccinated groups showed raised immunity from first vaccination. SNT microtiter and ELISA techniques showed highest RVF antibody titers induced by RVF vaccines at the 3rd month post first dose keeping the protective level till the 12th month. ELISA for SF vaccines showed the highest antibody levels against *P. multocida* (A, B6) and *M. hemolytica* (A) at the 4th month post first vaccination. But the 5th month was the highest for *P. multocida* (D) and *P. trehalosi* (T). There was no significant difference for the same antigen in the different vaccinated groups, but for them and the control group a significant difference was noticed. For the protection rate against *P. multocida* (A, B6 and D), a passive mouse protection test was used. The protection rate for A and B6 was 98%, and D was 93%. Concisely, the prepared combined inactivated RVF and SF vaccine is a potent providing sufficient protectivity against both diseases, relieving stressors on animals and workers.

KEYWORD:- RVF, ELISA, Pasteurella, Mannheimia, Vaccine, Shipping fever.

INTRODUCTION

Small ruminants are considered as a cornerstone with a great role contribute significantly to the country's economy through the entire system of production for both large and small farms. While skins, live animals, and carcasses make up a sizable amount of export revenue, meat and milk are important sources of protein. **(Abera and Mossie, 2023).** Over thirty percent of total domestic meat consumption comes from the production of goats and sheep **(Megra et al., 2006).** As any animal species, sheep and goats may be threaten by infectious diseases that may lead to huge economic losses and social bad effects especially in developing countries. Rift Valley fever and Shipping fever represent a viral and bacterial diseases affecting sheep and goat population dramatically.

A growing, significant zoonotic disease mostly affecting domestic ruminants in Africa. Rift Valley Fever (RVF) is spread by mosquitoes and causes substantial mortality rates in young animals, especially in cattle, sheep, camels, and goats, and causes enormous epizootics that result in miscarriages in infected female animals **(Al-Hazmi et al., 2003; Métras et al., 2012; Drake et al., 2013; Nanyingi et al., 2015).** That disease has serious harm to both human and animal health **(Pepin et al., 2010; Archer et al., 2013).**

RVF virus is the cause of the illness that is an acute fever arbovirus belonging to the Family Phenuiviridae, Genus Phlebovirus, Species Phlebovirus. Mostly found in sub-Saharan Africa, it occasionally produces epidemics in people and cattle. **(WHO, 2007; Archer et al., 2011; Aradaib et al., 2013; Sow et al., 2014).** The disease primarily affects domestic livestock (cattle, sheep, and goats), but it can also infect camels, buffaloes, and certain wild animals, which helps spread the virus **(Bird et al., 2009)**. The main vector is mosquito of the

genera *Aedes* and *Culex* **(Bird et al., 2009; Yousif et al., 2014).** The disease's ability to adapt to different environments and spread to nations with temperate climates poses a global public health issue. In numerous areas, outbreaks occur sporadically and are linked to factors such as socioeconomic status, hydrology, and climate **(Drake et al., 2013).** RVF historical range was restricted to sub-Saharan Africa. The worry about its spreading to other regions of the planet is exist **(Linthicum et al., 2007).** More specifically, since 1977, when RVF was first surfaced in the southern part of Egypt and then expanded to the Nile Delta, causing a serious outbreak in people and cattle. Egypt has seen periodic epidemics or epizootics **(Drake et al., 2013).** Nowadays, Egypt is the northernmost point of RVF. As expected, the spread of RVF in Egypt is unstable, leading to occasional outbreaks that most likely only happen when the virus is introduced in an environment with the proper entomologic and hydrologic circumstances **(Drake et al., 2013).** In fact, there is no particular cure for RVF, therefore vaccinating vulnerable animals in regions where the disease is common, like various African nations, Saudi Arabia, and Yemen, is still the most successful way to stop the virus from spreading among livestock and ultimately halt the transmission of the infection to humans **(Alhaj, 2016).** The effectiveness of a vaccination program relies on choosing the right vaccine, type, and timing based on the epidemiology of the disease being targeted **(OIE, 2018-a).** So that, the commercially available inactivated vaccines against RVF in animals, which were approved are used in the endemic areas **(Ikegami sand Makino, 2009; Faburay et al., 2017)**.

Shipping Fever is an acute respiratory illness that manifests ruminants following shipment that is referred to as "Shipping Fever". A severe fibrinous bronchopneumonia is the disease's hallmark, indicating that death usually happens early in the illness or at an acute stage. It's been common to refer to pneumonic pasteurellosis and pneumonic Mannheimiosis interchangeably **(Lopez and Martinson, 2017).** Pneumonia in sheep and goats can be resulted from a range of pathogens, such as bacteria, viruses, and parasites along with additional stress factors. In this context, bacteria have attracted interest due to their diverse clinical manifestations, the strong intensity of the illnesses, and the comeback of strains resistant to multiple chemotherapy drugs **(Nejiban and Al-Amery, 2018).** One of the main pathogens affecting small ruminants is Pasteurella species **(Daphal et al., 2018).** Typical organisms found in the tonsils and nasopharyngeal microflora of healthy sheep and goats include P. multocida, M. haemolytica, and B. trehalosi. Sheep and goats worldwide can suffer from cranioventral bronchopneumonia caused by small, pleomorphic, non-spore-forming gram-negative rod and coccobacilli bacteria. The primary cause of clinical infections in domestic animals by Pasteurella and Mannheimia species are the three main species: *M. haemolytica*, B. trehalosi, and *P. multocida* **(Abera and Mossie, 2023).** In this particular situation, *P. haemolytica*, biotype A, was reclassified as Mannheimia and placed in a different genus. On the other hand, *P. haemolytica* biotype T was first renamed as *P. trehalosi* (Bibersteina) **(Abera et al., 2014).** Pneumonic pasteurellosis can be caused by all types of *M. hemolytica* and *P. multocida* **(Mitku et al., 2017)**. The most effective methods for controlling these infections were antimicrobial drugs, but unfortunately, the widespread use of antibiotics increased the prevalence of drug-resistant strains of infection and diminished the efficacy of antimicrobial agents utilized in treating Pasteurella and Mannheimia infections **(Kehrenberg et al., 2001; Legesse et al., 2018).** Therefore, vaccination serves as a non-antibiotic preventative approach and is the most effective method of diseases control **(Jesse Abdullah et al., 2015). Sarwar et al., (2015)** found that the prepared hemorrhagic septicemia vaccine protection was extended to the 6th month while that Montanide adjuvanted vaccines and boosting of vaccinated animals with oil adjuvanted vaccine induced long live immunity that remained for more than 6 months. **Sotoodehnia et al., (2005)** cited that the prepared *P. multocida* (6: B) vaccine with Montanide ISA-70 adjuvant had 100% protection rate on days 24, 90 and 150 after the vaccination using the passive mouse protection test. **Jabbari and Moazeni Jula (2004)** also reported that, the Alum. adjuvanted hemorrhagic septicemia vaccine achieved 100% protection rate for the 1st 2 months, 83% for the 3rd and 4th months, 50% for the 5th month and finally 40% for the 6th month.

According to this fact, the most practical and cost-effective control strategy to save animal wealth in developing countries is vaccination **(Disassa et al., 2013; Mitku et al., 2017)**. Furthermore, numerous writers have recommended the utilization of powerful vaccines that are combined to protect against infectious diseases, ultimately saving time, energy, and money **(Ismail et al., 2023). Zaki et al. (2000)** reported that, there was no competition in the immune response when using a combined RVF, *M. haemolytica*, and *P. multocida* inactivated vaccine. There were no significant differences in serological and immunological test results between single and combined vaccines. All vaccinated animals developed protective levels of antibodies against RVF virus and Pasteurella strains.

So, this study was planned to prepare and evaluate a potent combined polyvalent inactivated vaccine against RVF and Shipping fever in sheep aiming to achieve a successful protective immunity against both diseases saving time cost and minimize the stressors.

MATERIALS AND METHODS

Ethical approval

Institutional Animal Care and Use Committee at Veterinary Serum and Vaccine Research Institute acknowledge the research manuscript and it has been

reviewed under our research authority and deemed compliance to bioethical standards in good faith.

Experimental animals
All the experimental animals were provided by the Veterinary Serum and Vaccine Research Institute (VSVRI) and Laboratory Animal House (LAH), Abbasia, Cairo, Egypt.

Swiss Albino baby mice
Ten mice (3 - 4 days old) were used in safety testing to guarantee total RVF virus inactivation.

Weaned swiss albino mice
One hundred and forty pathogenic-free Swiss albino mice, aged 21–28 days, were utilized to evaluate the immunological response (ED_{50}) of the produced vaccines.

Eight hundred and twenty Swiss albino weaned mice were grouped as follows; 810 were used for the passive mouse protection test and 10 were used for the Safety test for the shipping fever vaccines.
Lambs: Newborn healthy lambs of indigenous breeds (aged 7–10 days) were utilized in the safety test of RVF vaccines.
Sheep: Four to six months old twenty native breed sheep that had not been immunized before were utilized to assess the various vaccines that were prepared.

Used Strains for vaccine preparations
Viral strain
The original Rift valley fever virulent virus (RVF ZH501) was donated by the Rift Valley Fever Vaccine Research Department (RVFVRD); (VSVRI), Abbasia Cairo.

Bacterial strains
Serotypes A, B6 and D of *P. multocida*, *M. haemolytica* (A) and *P. trehalosi* (T) were provided with kindness by the Aerobic Bacterial Vaccine Research Department., VSVRI, ARC, Egypt.

The used adjuvant
Montanide™ ISA 206 VG (SEPPIC Co., France) was used as an adjuvant in the preparation of the vaccines.

Viral antigenic culture preparation
To boost the viral production, the RVF virus (ZH 501) was grown in BHK cells through three consecutive passages. while being monitored closely for biosafety. The virus was titrated using the microtiter technique in BHK cell culture in accordance with **Rossiter et al., (1985)** and the virus's titer was determined as $\log_{10}$ $TCID_{50}$/ml in accordance with **Reed and Muench, (1938).** According to **Bahnemann, (1975) and Mellencamp, (2004)**, the RVF virus was rendered inactive for 24 hours at 37˚C with binary ethylenimine (BEI) 1% of 0.1 M in 0.2 N NaOH solution to yield a final concentration of 0.001M BEI.

Bacterial antigenic culture preparation according to Ismail *et al*., (2023)
Each individual Pasteurella strain was cultured in nutrient broth that had been enhanced with yeast extract. The cultured samples were left to grow at 37 °C for 24 hours. Then inactivation with 0.5% formalin for 24 hours. The final vaccines formula was adjusted to contain 1×10^7 CFU for *P. multocida* (A, B6 and D), but 1×10^8 CFU for *M. haemolytica* (A) and *P. trehalosi* (T).

Vaccine preparations
Three different kinds of inactivated vaccines were prepared through this study as single RVF vaccine (Viral antigens), single Shipping fever vaccine (Bacterial antigens) and combined RVF and Shipping fever vaccine (Viral antigens and Bacterial antigens were mixed together in equal amounts). All of these vaccines were adjuvanted with Montanide™ ISA 206 VG according to the Montanide manufacturer (SEPPIC Co., France) utilizing a low shear rate and a regulated temperature of 31°C, at a 50% ratio while keeping the antigenic contents constant in all the prepared vaccines.

Quality control tests of the prepared vaccines
Sterility test
The vaccines produced were tested for sterility by using thioglycolate, soybean casein digest, Sabouraud dextrose agar medium, as well as mycoplasma solid and liquid media, following the recommendations of Code of **Federal Regulation (2005) and OIE (2018-b).**

Safety test
Viral safety test (Confirming the complete inactivation of RVF virus)
Confirmation of complete inactivation of RVF virus was performed to verify absence of any remaining pathogenic virus in the tissue culture **(OIE, 2018-a)** and in baby mice **(Randal et al., 1964)**, subjected for 10 days to monitor daily deaths. Mice that died during the first twenty-four hours were discarded as non-specific deaths.

Safety of RVF vaccines
Each of six lambs received Ten milliliters of the vaccines that have been prepared (with five milliliters for subcutaneous and five milliliters for intraperitoneal administration) (3 lambs/ vaccine) while the remained two lambs were kept as a control. For a period of two weeks, a daily clinical observation was conducted to identify any increase in body temperature or unusual clinical indications associated with renal failure.

Safety test for shipping fever vaccines
According to **Ismail et al., (2023)**, ten Swiss white Albino mice (5 for each vaccine) were injected with 0.2 ml of the prepared vaccines.

Potency test for RVF vaccines
In mice: Fivefold dilutions of each vaccine formula, ranging from 1:1 to 1:625 in appropriate medium, were prepared in accordance with **OIE (2018-a).**

Subsequently, a week apart, two injections of 0.2 ml of the vaccine I/P were given to each of five groups of adult mice (10 mice/group) (aged 21–28 days). Vaccinated mice were exposed to 0.1 ml of RVF challenge virus (10^3 $MIPLD_{50}$/ mouse inoculate) (I/P) seven days after the second dosage. In addition, there were two additional groups of mice: unvaccinated and not challenged, group maintained as control negative, while the other was challenged with RVF virus as a control positive. For duration of 21 days, all mouse groups were housed under close observation. The ED_{50} was determined as per **Reed and Muench (1938).**

Humeral immune response in sheep

Following the designed experimental work, the twenty sheep were used for evaluating the different prepared vaccine formulae where they divided into 4 groups (5sheep/group) housed in bug-friendly barns, given proper diet, sufficient hydration and necessary hygiene was maintained. The first group was vaccinated with single RVF vaccine (formula-1); the second group vaccinated with single SF vaccine (formula-2); the third group was vaccinated with the combined RVF-SF vaccine (formula-3) using a dose of 1 ml of each formula/animal inoculated S/C) administered in two doses with one month apart. The fourth group remained as the negative control without receiving any immunizations.

Sheep immune response to the tested vaccine formulae was monitored to RVF vaccine using the SNT microtiter technique where the antibody index was elaborated in accordance with (**EL Nimr 1980)** and ELISA **(voller et al., 1976),** monthly for up to a year post vaccination.

Potency test for shipping fever vaccine

Assessment of the immune response in the sheep vaccinated against Pasteurellosis was conducted by ELISA according to Voller *et al*., (1976). In addition, Passive mouse protection test was one for evaluation of the protection rate of the vaccinated sheep serum against challenge with the 3 strains of *P. multocida* (A, B6 and D) all over the intervals of the blood collection as described by **Alwis and Carter (1980).**

Statistical analysis

Findings were reported as an average plus or minus standard deviation (SD). SPSS program version 26 was utilized to conduct a One-way ANOVA test for determining the variance between groups (P values) (**IBM Corp., 2019**).

RESULTS

RVF Virus (ZH501) titration in Tissue Culture

On BHK cells, RVF virus clearly had a cytopathic effect (CPE) that resembles grape aggregation (rounding and aggregation in clusters). The viral titer progressively grew from 10^7 to $10^{7.5}$ $TCID_{50}$/ml in the 1st and 2nd passages to 10^8 $TCID_{50}$/ml in the 3rd passage. The found virus in the 3rd passage was used to prepare the vaccine.

Quality control tests of the prepared vaccines

Sterility test results

All the vaccines were passed the sterility testing and were found to be free from foreign contaminants, with no growth of mycoplasma, fungus, aerobic or anaerobic bacteria.

Safety of RVF vaccines

Inoculated baby mice did not show any abnormal post inoculation signs or deaths throughout the 10-day observation period and inoculated BHK cell culture revealed no CPE as depicted in the table (1).

Table (1): Safety test of inactivated RVF virus.

In BHK cell culture	In I/C inoculated baby mice			
No CPE	Number of mice	Mice showing illness	Dead mice	Survived mice
	10	0	0	10

** Evaluating the safety of the RVF vaccine that has been prepared showed that none of these vaccines induced any unfavorable side effects in the injected lambs.*

**Regarding the safety of Shipping fever vaccine formulae, all of them were assured to be safe; for 15 days of clinical observation, there were no reactions following injection either locally or systemically.*

Potency of RVF vaccine formulae

1) **In mice (ED50):** as shown in table (2), the different prepared vaccines showed an acceptable ED50/ ml limit (permissible limit 0.02 /ml).

Table (2): ED_{50}/ ml for different formulae of inactivated RVF vaccine.

RVF-ED_{50}/ml in inactivated vaccine formulae	
Single vaccine	Combined RVF-SF vaccine
0.0016	0.0015

2) **In sheep (Humeral Immune Response)**

SNT and ELISA revealed that the non-vaccinated sheep group displayed non-protective values, while the peak NI was recorded in the third month (3.7, 3.8) in groups (1) and (3) vaccinated with single RVF and combined vaccine respectively. The measured RVF neutralizing

antibodies remained within the protective limits until the 12th month after vaccination in both groups with values (1.5 and 1.9) correspondingly. Results of ELISA came in parallel to the results of SNT as shown in tables (3 and 4).

Table (3): Mean values of RVF neutralizing antibody indices in vaccinated sheep.

Time of sampling (MPV*)	Mean RVF antibody neutralizing indices in sheep vaccinated with		
	RVF vaccine (Group 1)	Combined vaccine (Group 3)	Control (Group 4)
Pre vaccination	0.370±0.036 [a]	0.323±0.040 [a]	0.317±0.015 [a]
1st	2.867±0.115 [b]	3.000±0.200 [b]	0.347±0.012 [a]
2nd	3.400±0.200 [b]	3.600±0.200 [b]	0.380±0.062 [a]
3rd	3.733±0.115 [b]	3.893±0.101 [b]	0.437±0.091 [a]
4th	3.533±0.115 [b]	3.733±0.115 [b]	0.360±0.208 [a]
5th	3.267±0.208 [b]	3.567±0.153 [b]	0.383±0.124 [a]
6th	3.167±0.058 [b]	3.357±0.140 [b]	0.377±0.137 [a]
7th	3.033±0.058 [b]	3.200±0.100 [b]	0.263±0.067 [a]
8th	2.767±0.153 [b]	2.933±0.115 [b]	0.343±0.111 [a]
9th	2.467±0.115 [b]	2.667±0.115 [b]	0.343±0.115 [a]
10th	2.233±0.153 [b]	2.467±0.115 [b]	0.237±0.015 [a]
11th	2.000±0.200 [b]	2.200±0.200 [b]	0.303±0.064 [a]
12th	1.533±0.115 [b]	1.967±0.289 [b]	0.300±0.052 [a]
13th	1.333±0.115 [b]	1.433±0.058 [b]	0.370±0.104 [a]

**MPV: month post vaccination.*

Data was described as mean ± SD. Data in the same row with varying lowercase letters were deemed statistically different at a p value of ≤0.05.

Table (4): Mean values RVF- ELISA optical density in vaccinated sheep.

Time of sampling MPV*	Mean RVF- ELISA optical density for sheep vaccinated with		
	RVF vaccine (Group 1)	Combined vaccine (Group 3)	Control (Group 4)
Pre vaccination	0.022±0.018[a]	0.043±0.008[a]	0.026±0.015[a]
1st	0.268±0.003[b]	0.275±0.004[b]	0.025±0.013[a]
2nd	0.282±0.003[b]	0.291±0.008[b]	0.025±0.017[a]
3rd	0.291±0.003[b]	0.303±0.009[b]	0.022±0.012[a]
4th	0.275±0.0134[b]	0.294±0.005 [b]	0.017±0.008[a]
5th	0.270±0.009[b]	0.282±0.011 [b]	0.016±0.006[a]
6th	0.265±0.004[b]	0.278±0.019 [b]	0.013±0.010[a]
7th	0.255±0.005[b]	0.271±0.010 [b]	0.014±0.010[a]
8th	0.246±0.005[b]	0.258±0.009 [b]	0.018±0.004[a]
9th	0.240±0.001[b]	0.245±0.004 [b]	0.030±0.014[a]
10th	0.236±0.001[b]	0.239±0.002 [b]	0.023±0.003[a]
11th	0.231±0.004[b]	0.237±0.002 [b]	0.023±0.002[a]
12th	0.226±0.002[b]	0.228±0.001 [b]	0.025±0.007[a]
13th	0.221±0.003[b]	0.224±0.001 [b]	0.021±0.005[a]

**MPV: month post vaccination.*

Data was described as mean ± SD. Data in the same row with varying lowercase letters were deemed statistically different at a p value of ≤0.05.

Shipping fever vaccines potency tests results

1) ELISA results for shipping fever vaccines (vaccines 2 and 3); tables (5 and 6) showed no significant difference between the two vaccinated groups in all months but there was a significant difference between the both vaccinated groups and the control group. In both vaccinated groups (vaccinated with single Shipping fever and the combined vaccines respectively), the antibody titers recorded an increase from the 1st dose of the vaccination and the booster dose, inducing a progress in the antibody titers reaching the highest level in the 4th month for *P. multocida* type A (1008.333±16.166, 986.333±4.163) and type B6 (955.000±39.611, 902.667±7.371) and *M. haemolytica* type A (870.000±31.607, 856.667±40.278). But the 5th month showed the highest titer for *P. multocida* type D (757.333±38.070, 701.333±2.517) and *P. trehalosi* type T (829.000±14.000, 799.667±13.796).

Table 5: Comparison between the shipping fever vaccines immunity against *P. multocida* (A, D, and B6) in sheep groups using ELISA test.

Time of sampling	*P. multocida*								
	A			B6			D		
	Shipping fever vaccine (Group 2)	The Combined vaccine (Group 3)	The control (Group 4)	Shipping fever vaccine (Group 2)	The Combined vaccine (Group 3)	The control (Group 4)	Shipping fever vaccine (Group 2)	The Combined vaccine (Group 3)	The control (Group 4)
Pre vaccination	19.667± 0.577	18.333± 2.082	19.000± 1.732	21.667± 1.528	19.333± 1.528	19.000± 1.732	17.667± 2.082	19.667± 2.517	19.333± 0.577
1st MPV	275.667± 24.173	241.667± 34.239	19.667± 1.528	256.667± 39.209	237.000± 14.107	19.333± 0.577	133.667± 34.530	127.000± 24.880	19.000± 1.732
2nd MPV	784.667± 38.760	766.667± 9.292	20.667± 1.155	578.000± 22.338	563.000± 17.578	20.000± 1.000	244.333± 31.880	215.000± 8.000	20.667± 2.082
3rd MPV	817.333± 17.502	797.667± 4.163	20.000± 1.000	785.000± 22.338	770.000± 17.578	19.000± 1.732	492.333± 4.163	474.333± 12.662	19.000± 1.732
4th MPV	1008.333± 16.166	986.333± 4.163	19.333± 2.082	955.000± 39.611	902.667± 7.371	20.667± 2.082	581.667± 30.089	565.667± 11.590	19.333± 0.577
5th MPV	841.000± 51.420	812.667± 11.060	19.667± 1.155	752.667± 12.503	732.667± 5.508	19.667± 1.528	757.333± 38.070	701.333± 2.517	20.000± 1.000
6th MPV	726.333± 45.490	691.333± 3.512	20.667± 2.082	568.667± 15.044	537.667± 15.503	19.000± 1.732	579.667± 28.095	551.333± 3.215	20.667± 2.082
7th MPV	618.000± 18.083	580.667± 18.583	19.667± 1.528	453.000± 11.136	436.667± 2.517	19.333± 0.577	451.667± 29.023	435.333± 15.948	19.667± 1.528
8th MPV	464.000± 6.557	451.000± 7.211	19.667± 0.577	389.667± 1.528	371.000± 12.530	20.667± 2.082	370.667± 3.215	358.000± 8.544	19.000± 1.732
9th MPV	382.000± 5.568	373.667± 1.155	20.667± 2.082	340.000± 9.644	324.667± 9.713	20.000± 1.000	232.000± 33.601	208.667± 8.505	20.667± 2.082
10th MPV	331.667± 5.033	322.000± 4.583	20.000± 1.000	307.333± 2.517	291.667± 10.408	20.667± 2.082	174.333± 1.155	165.667± 7.572	19.333± 0.577
11th MPV	291.333± 19.140	267.000± 7.550	19.667± 1.528	219.000± 8.000	195.000± 14.422	19.000± 1.732	137.333± 10.017	125.333± 4.933	20.000± 1.000
12th MPV	217.000± 2.000	207.333± 6.658	19.333± 0.577	166.000± 4.583	154.000± 6.557	19.333± 0.577	102.000± 5.292	91.000± 5.292	20.667± 2.082
13th MPV	141.000± 3.464	132.000± 6.245	19.333± 0.577	91.333± 5.132	79.333± 6.658	20.000± 1.000	46.333± 1.155	34.333± 8.145	19.000± 1.732

Group-2 vaccinated with single SF vaccine
Group-3 vaccinated with combined RVF-SF vaccine
Group-4 unvaccinated group
**MPV: month post vaccination.*
Data was described as mean ± SD. Data within the same row with different small letters were considered significantly different at p value ≤0.05.

Table (6): Comparison between the Shipping fever vaccine immunity against *M. haemolytica* (A) and P. trehalosi (T) in sheep groups using ELISA test.

Time of sampling	*M. haemolytica (A)*			*P. trehalosi (T)*		
	Shipping fever vaccine (Group 2)	The Combined vaccine (Group 3)	The control (Group4)	Shipping fever vaccine (Group 2)	The Combined vaccine (Group 3)	The control (Group4)
Pre vaccination	18.667±2.517	18.667±2.082	19.000±1.732	18.667±1.528	19.667±0.577	19.000±1.732
1st MPV	262.333±31.533	213.000±12.000	19.667±1.528	135.667±16.042	129.667±11.015	19.667±1.528
2nd MPV	575.667±14.295	550.667±12.014	20.667±1.155	319.333±16.862	300.000±15.620	19.667±1.155
3rd MPV	743.000±30.050	727.667±34.269	20.000±1.000	622.667±13.204	601.000±15.620	19.000±1.732
4th MPV	870.000±31.607	856.667±40.278	19.333±2.082	793.333±4.726	774.000±12.166	20.667±1.155
5th MPV	864.667±8.386	837.667±15.948	19.667±1.155	829.000±14.000	799.667±13.796	20.000±1.000
6th MPV	726.000±9.849	697.333±16.503	20.667±2.082	692.667±5.508	675.667±10.066	19.333±0.577
7th MPV	516.667±15.631	512.000±6.557	19.667±1.528	471.667±18.502	444.000±3.606	20.000±1.000
8th MPV	429.667±3.055	416.667±8.737	19.667±0.577	418.333±2.309	393.667±16.803	19.667±0.577

9th MPV	356.333±14.295	335.000±7.211	20.667±2.082	342.000±10.149	321.333±9.609	20.667±2.082
10th MPV	293.667±3.055	276.667±11.150	20.000±1.000	290.667±6.028	272.333±11.590	19.333±0.577
11th MPV	253.333±6.506	230.667±13.650	19.667±1.528	240.667±7.506	226.333±6.429	19.667±1.528
12th MPV	193.667±4.619	178.000±9.849	19.333±0.577	105.000±7.211	91.667±6.110	19.667±1.528
13th MPV	145.667±2.309	132.333±8.622	19.333±0.577	55.667±2.309	40.667±10.214	19.333±0.577

Group-2 vaccinated with single SF vaccine
Group-3 vaccinated with combined RVF-SF vaccine
Group-4 unvaccinated group
**MPV: month post vaccination.*
Data was described as mean ± SD. Data within the same row with different small letters were considered significantly different at p value ≤0.05

2) Passive mouse protection test results

As tabulated in table (7), the overall means protection test results in the vaccinated sheep groups (group 2 and 3) serum in comparison to the control one, challenged with *P. multocida* (A, D and B6) virulent strains were as follow; 98% protection for all vaccinated groups but the control group had 0% protection for A and B6. But, for type D, the protection was 93% for the vaccinated groups and 0% for the control group.

Table (7): Passive mouse protection test results in sheep groups challenged with virulent strains of *P. multocida* type A, D and B6.

Time intervals	***P. multocida* virulent strains**																	
	A						**B6**						**D**					
	Shipping fever vaccine (Group 2)		**The Combined vaccine (Group 3)**		**Control (Group 4)**		**Shipping fever vaccine (Group 2)**		**The Combined vaccine (Group 3)**		**Control (Group 4)**		**Shipping fever vaccine (Group 2)**		**The Combined vaccine (Group 3)**		**Control (Group 4)**	
	dead	**live**	**dead**	**live**	**dead**	**live**	**dead**	**live**	**dead**	**live**	**dead**	**live**	**dead**	**live**	**dead**	**live**	**dead**	**live**
Pre vaccination	10	0	10	0	10	0	10	0	10	0	10	0	10	0	10	0	10	0
1st MPV	1	9	1	9	10	0	1	9	1	9	10	0	2	8	2	8	10	0
2nd MPV	0	10	0	10	10	0	0	10	0	10	10	0	1	9	1	9	10	0
3rd MPV	0	10	0	10	10	0	0	10	0	10	10	0	0	10	0	10	10	0
4th MPV	0	10	0	10	10	0	0	10	0	10	10	0	0	10	0	10	10	0
5th MPV	0	10	0	10	10	0	0	10	0	10	10	0	0	10	0	10	10	0
6th MPV	0	10	0	10	10	0	0	10	0	10	10	0	0	10	0	10	10	0
7th MPV	0	10	0	10	10	0	0	10	0	10	10	0	1	9	1	9	10	0
8th MPV	1	9	1	9	10	0	1	9	1	9	10	0	2	8	2	8	10	0
Overall means Protection %	98		98		0		98		98		0		93		93		0	

Group-2 vaccinated with single SF vaccine
Group-3 vaccinated with combined RVF-SF vaccine
Group-4 unvaccinated group
**MPV: month post vaccination.*
Protection % = No. of survived mice X 100
Total No. of mice

DISCUSSION

The capability of the immune system to react swiftly to pathogens is greatly improved by vaccines, often known as "weapons of mass protection," after a second episode of resistance (**Cohen and Marshall, 2001; Curtiss, 2002**). Activating a strong, sustained immune response to the given antigen is the goal of immunization. To meet these goals, it is necessary to utilize efficient adjuvant and vaccine methods that can enhance the vaccine's immunogenicity enough to trigger a strong immune response. (**Fearon, 1997; Bomford, 1998**).

In this research, Rift Valley Fever (RVF) and Shipping Fever inactivated vaccines were prepared as single vaccines and a combined one of them adjuvanted with Montanide™ ISA 206 VG.

The primary goal of this project is to create a strong, combined vaccine that can effectively safeguard sheep against RVF and SF without antagonizing effect between the used antigens and minimize stress of repeating handling and injection of animals, easier for workers, in addition to reduce required time and cost. All of these

goals were in agreement with **Awaad (2004)** who cited that the combined vaccines can relieve animals from different stressors of multiple injections of different mono vaccines.

Concerning titration of RVF virus in BHK cells, it was found that the virus had a titer of 10^8 $TCID_{50}$/ml with CPE represented by rounded and aggregated cells **(Billecocq et al., 1996).** Complete virus inactivation was confirmed in mice and tissue culture (Table 1) for 14 days post-inoculation where there were no clinical abnormalities or fatalities in mice and no observed CPE in cell cultures in accordance of the **Code of Federal Regulations (2005)**.

Testing the prepared RVF and SF vaccines showed they were clean of foreign contaminants (bacteria, fungi, mycoplasma) and safe with no adverse reactions in lambs after vaccination as evidenced by the fact that the lambs body temperatures did not rise after the vaccination and remained within normal ranges for the next 14 days without any abnormalities or fatalities. These results align with the suggestions of the **British Veterinary Pharmacopoeia (2013)** and agreed with **Wassel et al. (1996) and Code of Federal Regulations (2005)** who suggested that the ultimate vaccine should be safe for animals to use and free of outside pollutants In addition, the ED_{50} of the inactivated RVF virus was found to be 0.0015 ED_{50}/ml in mice coming supported by **Randal et al., (1964)** who used the ED_{50} test to evaluate the inactivated vaccine, as it should not be more than 0.02 milliliters per mouse according to the **WHO (1983)** recommendation**.**

Monitoring the immunity response of vaccinated sheep groups 1 and 3 with single RVF inactivated vaccine and the combined RVF and Shipping fever vaccine respectively as declared in table (3), it was found that the RVF serum neutralizing antibody titer in group (3), reached (3.000±0.200) by the first MPV whereas in group (1), it reached (2.867±0.115). Both groups showed an increase in their antibody levels recording their peaks by the 3rd MPV (3.733±0.115 in group-1 and 3.893±0.101 in group-3). Both sheep groups remained inside the protective RVF antibody level till end of 12th MPV with levels 1.533±0.115 in group-1 and 1.967±0.289 in group-3. These findings were backed up by the data collected by **Daoud et al., (2001)**, as they found the same thing using the FMD/RVF combined vaccination: where there was no discernible variation in the immune system's reaction provoked by the combined FMD and RVF.

The ELISA results showed in table (4) occur simultaneously with findings from SNT (**Hendriksen et al., 1998**).

Wells et al., (1984) outlined how the polyvalent clostridia and *P. haemolytica* vaccine had a protective effect against the infection, so that the multiple antigens did not conflict and the protection effect of the polyvalent vaccine and the several antigens content did not adverse the effect of each antigen.

Also, for estimating the induced immunity by the prepared vaccines against shipping fever, ELISA technique was used. The results have been shown in tables (5 and 6) demonstrated no notable distinction between the two groups that received the vaccine (group-2 vaccinated with single SF vaccine and group-3 vaccinated with the combined RVF-SF vaccine) all over the experimental period with a major distinction among the groups that received vaccines and the unvaccinated control (group - 4). The antibody levels in both groups that were vaccinated recorded an increase from the 1st month after vaccination and the booster 2nd dose resulting in a progress in the antibody titers reaching the highest levels in the 4th month for *P. multocida* type A (1008.333±16.166, 986.333±4.163); for type B6 (955.000±39.611, 902.667±7.371) and for *M. haemolytica* type A (870.000±31.607, 856.667±40.278). But the 5th month recorded the highest for *P. multocida* type D (757.333±38.070, 701.333±2.517) and *P. trehalosi* type T (829.000±14.000, 799.667±13.796). In this respect, **Abd El-Moneim et al., (2022),** used ELISA for evaluating their prepared vaccine against pasteurellosis in sheep and they cited that, for *P. multocida* (A and D) the 5th month was the higher in antibodies titers (550, 554 respectively), but for *P. multocida* type B6 was higher in the 4th month (710). For *M. haemolytica* (A) and *P. trehalosi* (T) the 5th month was the higher (745, 650 respectively). **Pesca *et al.*, (2020)** evaluated the immune response acquired from their prepared *M. haemolytica* vaccine by ELISA test and concluded that the vaccination of ewes and offspring with *M. haemolytica* vaccine provides a good protection. With regard to the use of combined Pasteurella and viral vaccines, **Alemnew et al., (2022)** prepared a combined Pasteurellosis and Peste des Petits Ruminants vaccines and proved that the vaccine was effective in reducing the animal's morbidity and mortality. **Muenthaisong et al., (2021)** prepared a combined Foot-and-mouth disease (FMD) and Haemorrhagic septicemia (HS) vaccine and concluded that, there were no significant differences statistically between HS vaccinated and FMD-HS combined vaccinated groups without any antigenic antagonization. By using ELISA test, **Mori et al., (2020)** evaluated their prepared combined polyvalent vaccine against *P. multocida, H. somni* and *M. haemolytica* and concluded that the booster dose had raised the titers of antibodies. **El-Kattan et al., (2019)** used Indirect Hemagglutination (IHA) test to estimate the acquired immunity from vaccination with polyvalent *P. multocida* and *M. haemolytica* types. They found after the vaccination that, for *P. multocida* type B, the 2nd month was the higher month in antibodies titers, the 2nd and 3rd months were the higher for *P. multocida* (D) and *M. haemolytica* (A) in antibodies titers, the 3rd month was the higher month in antibodies titers for *M. haemolytica* type T and the 5th month was the higher month in

antibodies titers for *P. multocida* type A. **Rahman et al., (2016)** used field isolates of *P. multocida* for preparation of Pasteurella vaccine. For evaluating their prepared vaccine, they used IHA test and concluded that the vaccine may be protective against hemorrhagic septicemia. **Tanwar et al., (2016)** used ELISA test for comparing between inactivated *P. multocida* with Aluminum-based mineral salts adjuvant and Herbal adjuvant. They found that the immunity developed from the vaccine with Alum gel adjuvant raised from the 4th up to 14th weeks. But, the herbal adjuvanted vaccine the immunity raised from the 3rd month to the 4th month then, began to decrease. While **Sarwar et al., (2015)** found that the protection was extended to the 6th month who prepared a hemorrhagic septicemia vaccine and evaluated with IHA test concluded that Montanide adjuvanted vaccines and boosting of vaccinated animals with oil adjuvanted vaccine developed long lasting immunity that lasted beyond 6 months. Our results agree with **Hanna et al., (2014)** who prepared a Pasteurella and clostridia combined vaccine and used ELISA for the evaluation. Their results agreed with us as the antibodies developed against Pasteurella strains were the highest level between the 3rd and 4th months.

Also, Passive mouse protection test was used in this study to assess the effectiveness of the Shipping fever (*P. multocida*; A, B6 and D) vaccines. As demonstrated in table (7), for single and combined Shipping fever vaccines, the overall means protection test results showed that, for *P. multocida* (A and B6) the protection rate was 98% and for *P. multocida* (D) was 93%. While for the control group, the protection rate was 0%. On the same way, **Jaffri et al., (2006)** used the passive mouse protection test and IHA test to compare between hemorrhagic septicemia vaccine adjuvanted with oil and the adjuvanted with alum-precipitate vaccine. The passive mouse protection test gave 20% protection for the alum precipitated vaccine, while 60% protection for the oil adjuvanted vaccine. On the other side, by using IHA test, the result showed that the vaccine was adjuvanted with alum precipitate gave antibodies protective titer for 4 months. While the vaccine was adjuvanted with oil gave antibodies protective titer for 10 months. These results confirmed that the vaccine adjuvanted with oil give long live protection and the booster dose is important for producing long solid immunity. **Sotoodehnia et al., (2005)** used the passive mouse protection test to evaluate the prepared *P. multocida* (6: B) vaccine with Montanide ISA-70 adjuvant and the resulted protection rate was 100% on days 24, 90 and 150 after the vaccination. **Jabbari and Moazeni Jula (2004)** used the Alum. adjuvant for preparing hemorrhagic septicemia vaccine and reported that the protection rate for this vaccine was 100% for the 1st 2 months, 83% for the 3rd and 4th months, 50% for the 5th month and finally 40% for the 6th month. So, the combined vaccine could be an effective vaccine against both diseases.

CONCLUSION

Based on the findings, it may be inferred that, the combined polyvalent inactivated RVF and Shipping fever vaccine adjuvanted with Montanide^{TM} ISA 206 VG is a safe potent vaccine able to provide sheep with good levels of specific immunity enable them to resist both diseases.

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