CALCIUM PHOSPHATE AS AN ADJUVANT FOR INACTIVATED RIFT VALLEY FEVER VACCINE

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Abstract

Vaccination to protect animal against infectious diseases may be enhanced by using adjuvants that can selectively stimulate immunoregulatory responses. A novel adjuvant composed of calcium phosphate (CAP) was compared with the commonly used aluminium hydroxide (alum) gel adjuvant for its ability to induce immunity to Rift Valley Fever virus. Results indicated that CAP was more potent as an adjuvant than alum, induced high titres of antibody. Furthermore, it is economical, simple to manufacture and it is a natural constituent of the animal body.

INTRODUCTION

Rift Valley Fever (RVF) is an arthropod-borne viral disease affecting animals and human. It is an economically important viral disease and widely distributed in different localities of Africa and Asia where periodic epizootic and epidemic occurred causing heavy losses among lambs and calves (Woods *et al.,* 2002 and Fagbo, 2002).

RVF disease is caused by RNA single stranded virus belonging to family Bunyaviridae (WHO, 1982 and Connie, 1996). The appearance of RVF disease in Egypt in 1977 (Imam *et al.,* 1977), and its reappearance in 1993 (El-Gabery *et al.,* 1994) increased the demand to develop a potent inactivated RVF vaccine. The adjuvants are modulators of the immune system and their function is to improve vaccine efficacy in order to offer protection against infection. One of these adjuvants is calcium phosphate which is a novel adjuvant elicited little or no inflammation at the site of inoculation and induced higher titre of neutralizing antibody (Bio Sante Pharmaceuticals Inc., 2000). Alum compounds are the most extensively used adjuvants in licensed vaccines. Although they effectively enhance immune response, they cause severe inflammatory reaction at the injection site and the duration of this inflammation is somewhat long as cited by Goto *et al.* (1997) who reported that the local tissue reactions caused by injection of CAP gel completely ceased by the 4th week, while irritation caused by aluminium hydroxide gel persisted for 8 weeks.

The aim of this work is to study the effect of CAP (as an adjuvant) when added to Rift Valley Fever binary inactivated virus on the immune response of vaccinated sheep.

MATERIALS AND METHODS

1. Animals

1.1. Mice (Swiss albino mice)

1.1.1. Adult mice

Twenty one (twenty eight - day old) mice were used for toxicity and potency test for both calcium phosphate and vaccines, respectively.

1.1.2. Baby mice

Three (five- day old) mice were used for safety of the prepared inactivated virus.

1.2. Sheep

1.2.1. Fourteen susceptible balady sheep six month age were used for the potency test of the vaccines.

1.2.2. Eight lambs (5-10- day old) were used for safety of the RVF vaccine with different calcium phosphate concentrations.

2. Virus

RVF virus ZH-501 with a titre of 7.5 $\log_{10}$ $TCID_{50}$/ml were kindly supplied by RVF Department, Veterinary Serum and Vaccine Res. Inst., Abbasia, Cairo. It was isolated from human patient in Zagazig, Sharquia Province during outbreak in 1977.

3. Conjugate

Horseradish peroxidase conjugate labeled antispecies (antisheep) was purchased from Sigma Company. It was diluted in PBS immediately before use for ELISA test.

4. Adjuvant

1.1.1. Aluminium hydroxide gel

2 % gel was purchased from Honil Limited, London, United Kingdom.

1.2.1. Calcium phosphate (CAP)

Composed of:

1. Calcium chloride (Winlab).
2. Dibasic sodium phosphate (El-Nasr Pharmaceutical Chemicals Co.).
3. Sodium citrate (Analar).

It was prepared according to Bio Sante Pharmaceuticals, Inc., Smyrna, Georgia (2000).

Toxicity test

Adult mice were used for the toxicity of CAP adjuvant in vaccine preparation. Three groups of mice (15 per each), one inoculated I/P and the second S/C while, the third group was kept as a control, and all groups were observed for 10 days post-inoculation.

Preparation of the vaccine

1. Virus

RVF ZH-501 was inactivated by binary ethyleneimine according to M, Eman (1995), then different forms of vaccines were prepared, one with 25 % aluminium hydroxide gel and the three others with 75 %, 50 % and 25 % calcium phosphate, respectively.

2. Addition of CAP adjuvant

CAP was added with different concentrations to the inactivated virus as 75 %, 50 % and 25 %.

Evaluation of the vaccine

1. Sterility test

It was done according to OIE (2000).

2. Safety test

It was performed according to El-Nimr (1980) and M, Eman (1995).

a. Baby mice

They were inoculated I/C.

b. Lambs

Nine lambs were inoculated each with 10 ml of the vaccine (5 ml I/P and 5 ml S/C), then these animals were observed for 10 days for any sign of RVF disease or death (El-Nimr, 1980 and Eman, 1995).

Potency test

Adult mice were inoculated I/P by two doses of the vaccine one week apart, and then challenged to calculate the ED_{50} for each formula of the vaccine separately according to Randall *et al.* (1964).

Experimental Design

Fourteen balady sheep were divided into 5 groups:

Group (1): Three sheep were vaccinated S/C with inactivated RVF vaccine with 75 % CAP.

Group (2): Three sheep were vaccinated S/C with inactivated RVF vaccine with 50 % CAP.

Group (3): Three sheep were vaccinated S/C with inactivated RVF vaccine with 25 % CAP.

Group (4): Three sheep were vaccinated S/C with inactivated RVF vaccine with

aluminium hydroxide gel (commercial one).

Group (5): Two sheep were kept as control (non-vaccinated).

All animals were observed for 6 months post- inoculation for sero-conversion.

Serological tests

1. Serum neutralization test

It was done according to Walker (1975).

2. Indirect enzyme linked immunosorbent assay (indirect ELISA)

It was done according to Voller *et al.* (1976).

RESULTS AND DISCUSSION

Table 1. Results of toxicity test in mice.

Adjuvant	Mice		Control
	S/C	I/P	
CAP	0/15*	0/15*	0/15*

* Number of dead mice over number of survived mice.

Table 2. Results of sterility, safety and potency test of the prepared vaccine.

Type of vaccine	Sterility	Safety		Potency*
		Baby mice**	Lamb***	ED_{50}/ml
CAP 75 %	Sterile	0/8	0/2	0.0006/ml
CAP 50 %	Sterile	0/8	0/2	0.0003/ml
CAP 25 %	Sterile	0/8	0/2	0.0005/ml
Aluminium hydroxide gel	Sterile	0/8	0/2	0.0006/ml

*The minimum permissible limit of ED_{50}/ml is 0.02 ml.

** Safety test in baby mice performed of inactivated RVF virus without adjuvant.

*** Safety in lamb = No thermal or clinical reaction or manifestation.

Table 3. Result of neutralizing antibody index (NI) of sheep sera vaccinated with different formula of RVF prepared vaccines.

Group of animals	Types of different adjuvant	NO. of animals	Before Vaccination	Neutralizing Indices								
				Weeks post Vaccination								
				1	2	3	4	8	12	16	20	24
G1	CAP 75 %*	3	0.4	1.0	1.4	1.7	2.4	2.7	3.0	2.6	2.0	1.5
			0.3	0.7	1.0	1.4	1.7	2.0	2.4	2.5	2.1	1.7
			0.3	0.7	1.0	1.7	2.0	2.4	2.7	2.5	2.5	1.8
		Mean	0.33	0.8	1.1	1.6	2.0	2.4	2.7	2.5	2.2	1.6
G2	CAP50%*	3	0.4	0.7	1.4	1.7	2.4	2.7	3.0	3.3	3.7	3.4
			0.3	1.4	1.7	2.0	2.7	3.0	3.4	3.7	4.0	4.0
			0.4	1.0	1.4	2.0	2.4	3.0	3.2	3.4	3.7	3.7
		Mean	0.36	1.0	1.5	1.9	2.5	2.9	3.2	3.5	3.8	3.7
G3	CAP 25 %*	3	0.4	0.7	1.4	1.7	2.0	2.4	2.5	3.0	2.7	2.5
			0.4	0.7	1.4	1.7	2.0	2.4	2.7	3.7	3.0	2.9
			0.3	1.0	1.0	1.7	2.4	2.7	3.0	3.7	3.4	2.9
		Mean	0.36	0.8	1.2	1.7	2.13	2.5	2.7	3.4	3.0	2.8
G4	Alum gel	3	0.4	0.7	1.4	1.7	2.0	2.4	2.4	2.0	1.7	1.4
			0.3	1.0	1.0	1.4	1.7	2.0	2.7	2.7	2.0	1.7
			0.4	0.7	1.4	1.7	2.0	2.7	2.7	2.7	2.4	1.7
		Mean	0.36	0.8	1.2	1.6	2.0	2.3	2.6	2.3	2.0	1.6
G5	control	2	0.3	0.2	0.4	0.3	0.3	0.2	0.3	0.3	0.2	0.3
			0.1	0.3	0.2	0.3	0.4	0.2	0.2	0.3	0.3	0.3
		Mean	0.2	0.25	0.3	0.3	0.35	0.2	0.25	0.3	0.25	0.3

G1: Binary inactivated RVF vaccine with 75 % of CAP

G2: Binary inactivated RVF vaccine with 50% of CAP

G3: Binary inactivated RVF vaccine with 25 % of CAP

G4: Binary inactivated RVF vaccine with alum hydroxide gel

G5: Control (non- vaccinated).

* CAP: Calcium phosphate

Table 4. Result of indirect Elisa technique of sheep sera vaccinated with different formula of RVF prepared vaccines

Group of animals*	Types different adjuvant**	NO. of animals	Before Vaccination	Optical Density								
				Weeks post Vaccination								
				1	2	3	4	8	12	16	20	24
G1	*CAP 75 %	3	0.020	0.057	0.062	0.074	0.088	0.096	0.110	0.100	0.094	0.086
			0.010	0.051	0.059	0.062	0.076	0.088	0.103	0.094	0.082	0.078
			0.012	0.044	0.060	0.069	0.080	0.090	0.114	0.101	0.098	0.075
		Mean	0.014	0.050	0.060	0.068	0.081	0.091	0.109	0.098	0.091	0.079
G2	CAP 50 %*	3	0.011	0.055	0.065	0.079	0.084	0.096	0.110	0.134	0.145	0.144
			0.020	0.062	0.069	0.082	0.096	0.102	0.131	0.141	0.158	0.153
			0.017	0.059	0.062	0.078	0.089	0.102	0.127	0.139	0.150	0.148
		Mean	0.016	0.058	0.065	0.079	0.089	0.100	0.122	0.138	0.151	0.148
G3	CAP 25 %*	3	0.011	0.056	0.061	0.076	0.086	0.097	0.117	0.122	0.117	0.110
			0.021	0.055	0.063	0.069	0.081	0.091	0.112	0.128	0.125	0.121
			0.17	0.061	0.064	0.071	0.079	0.101	0.113	0.125	0.119	0.107
		Mean	0.016	0.057	0.062	0.072	0.082	0.096	0.114	0.125	0.120	0.112
G4	Alum gel	3	0.021	0.055	0.057	0.067	0.074	0.089	0.104	0.095	0.089	0.077
			0.013	0.066	0.059	0.069	0.071	0.086	0.111	0.087	0.077	0.070
			0.014	0.051	0.061	0.071	0.078	0.090	0.107	0.090	0.081	0.075
		Mean	0.016	0.057	0.059	0.069	0.074	0.088	0.107	0.090	0.082	0.074
G5	control	2	0.011	0.012	0.010	0.018	0.019	0.013	0.020	0.012	0.020	0.018
			0.020	0.017	0.021	0.019	0.022	0.017	0.011	0.010	0.011	0.017
		Mean	0.015	0.014	0.015	0.018	0.020	0.015	0.015	0.011	0.015	0.017

G1: Binary inactivated RVF vaccine with 75 % of CAP

G2: Binary inactivated RVF vaccine with 50 % of CAP

G3: Binary inactivated RVF vaccine with 25 % of CAP

G4: Binary inactivated RVF vaccine with alum hydroxide gel

G5: Control non- vaccinated

* CAP: Calcium phosphate - cut – off value= 0.03

Fig. (1): Result of neutralizing antibody index (NI) of sera of sheep vaccinated with different forms of RVF prepared vaccines

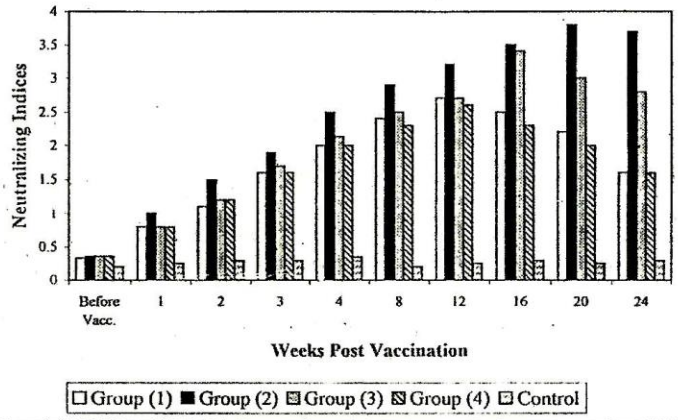
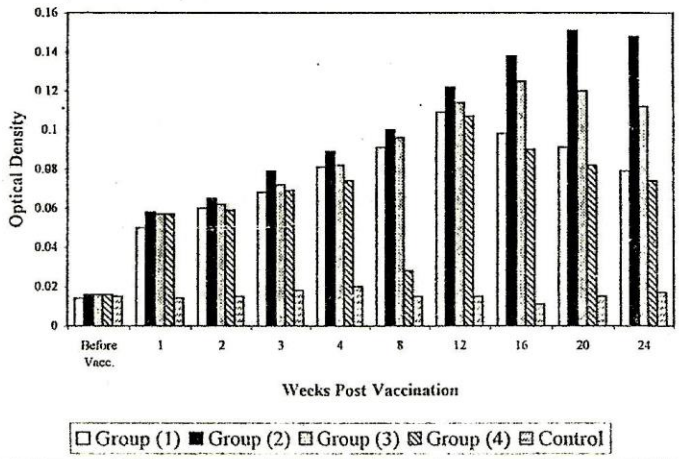


Fig. (2): Result of indirect ELISA technique of sera of sheep vaccinated with different forms of RVF prepared



When the toxicity test was carried out on adult mice, the result revealed that neither S/C nor I/P routes of injection elicited inflammation at the site of injection or any sign of toxicity during the test as shown in Table 1. All batches of the prepared vaccines were sterile and safe when inoculated in baby mice and lambs which showed no variation of body temperature of lambs or no signs of illness and no deaths were observed in mice and lambs. The most potent vaccine was that containing 50 % calcium phosphate as an adjuvant as its ED_{50}/ml was 0.0003/ml followed by vaccine containing 25 % CAP as an adjuvant as its ED_{50}/ml was 0.0005/ml and the last one was 75 % calcium phosphate as its ED_{50}/ml was 0.0006/ml as shown in Table 2. All these batches were within the permissible limit as cited by Randall *et al.* (1964) who said that the ED_{50} must not be more than 0.02/ml. The ED_{50} of aluminium hydroxide gel vaccine batch was 0.0006/ml. These results agreed with K, Gehan (1990) and M, Eman (1995), when they used alum gel vaccine as there is no available data on RVF vaccine adjuvated with calcium phosphate.

The immune response of vaccinated sheep was tested by SNT (Table 3 and Figure 1) and showed the neutralizing indices of all groups of sheep. It was noticed and sera of sheep vaccinated with RVF vaccine with 50 % calcium phosphate gave the highest level at second week (mean of NI equal 1.5) as Pini *et al.* (1973) suggested that the protective titre was 1.5 $\log_{10}$ $TCID_{50}$/ml and reached its peak at 20th week post vaccination with a mean of NI 3.8. This agreed with Biosante Pharmaceuticals Inc. (2000) who found that Herp's simplex virus type two (HSV-2) plus calcium phosphate gave high antibody level at 6 weeks after immunization and still was high up to the week fourteen. In case of RVF vaccine with 25 % CAP adjuvant, the protective level appeared at 3rd week post-vaccination with an average of 1.7 NI, while RVF vaccine with 75 % adjuvant, the protective level appeared at post vaccination with an average of 1.6 NI and reached its peak at the 16th week post vaccination being 3.4 NI. The protective level of the vaccine of aluminium hydroxide gel appeared at 3rd week post vaccination with an average 1.6 NI and reached its peak at 12th week post vaccination being 2.6 NI.

The result of SNT was correlated with that obtained by ELISA test as shown in Table 4 and Figure 2. This agreed with M, Eman (1995) and Hassan *et al.* (2001), but they used inactivated Rift Valley Fever inactivated aluminium hydroxide gel vaccine.

From the previous data, CAP adjuvant induces no inflammation at site of entry and induces immunological enhancement without toxicity and gives a higher titer of antibody earlier than aluminium hydroxide gel, furthermore, it is easy to manufacture on an industrial scale and shows insignificant variation in quality and physicochemical properties between batches in production condition.

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إستخدام فوسفات الكالسيوم كمحسن مناعى للقاح حمى الوادى المتصدع المثبط

خيرات عبد المجيد عليان ، للى صبحى سلامة ،
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إن التحصين هو الوسيلة لوقاية الحيوانات ضد الأمراض الوبائية ويمكن رفع المستوى المناعى للحيوانات بإضافة بعض المحسنات للقاحات. وقد استخدم محسن جديد فى هذا البحث وهو فوسفات الكالسيوم وبمقارنته بالالومنيوم هيدروكسيد جل فى هذا البحث وجد أنه أقوى ويعطى أجسامًا مناعية بنسبة أعلى من الالومنيوم هيدروكسيد جل. بالإضافة إلى أنه مادة أقتصادية وسهلة التصنيع كما أنها أحدى المكونات الطبيعية فى جسم الحيوان.