TRIALS FOR USING SKIMMED MILK AS A STABILIZER FOR ATTENUATING RIFT VALLEY FEVER VIRUS VACCINE

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(Manuscript received 9 March 2004)

Abstract

In this study, the Rift Valley Fever virus attenuated vaccine was used after addition of skimmed milk as stabilizer with different concentrations (10%, 15% and 20%). After lyophylization, it was found that there was no difference between all the concentrations except that 15% and 20% had no good physical appearance; 10% concentration was chosen to continue this study. Regarding the titration and evaluation of the prepared vaccine, it was noticed that the titre was $10^{7.6}$ $TCID_{50}$ /ml. It was found sterile and safe in hamster and sheep. The potency test in tissue culture was $10^{7.6}$ $TCID_{50}$ /ml and the mean SNT of sheep sera was 80. The titre at room temperature was $10^{7.3}$ TCID 50 / ml on 1st day and reached $10^{7.6}$ $TCID_{50}$ /ml on 7th day, while in 4°C the titre was $10^{7.6}$ $TCID_{50}$ /ml in 1st month reaching to $10^{4.6}$ $TCID_{50}$ / ml in 12th month, while, in –20°C the titre was not changed up till 9th month and reached $10^{6.3}$ $TCID_{50}$ /ml at 12th month. By estimating the level of antibodies of the prepared vaccine, it was high up till the end of the experiment (6th month) with an average of 3.18 NI by using serum neutralization test. From this study, it is clear that 10% skimmed milk is the best concentration stabilizer of choice, the prepared attenuated vaccine and being suitable for use when stored at room temperature for 48 hours and 6 months at 4 °C. It can be stored at –20 °C without loss in its titre till 9 months.

INTRODUCTION

Rift Valley Fever (RVF) is an acute febrile mosquito borne viral disease affecting animals and human. It is an economically important viral disease and widely distributed in different localities of Africa where periodic epizootic and epidemic occurred causing heavy losses among lambs and calves and abortion of pregnant animals. RVF disease is caused by an RNA single stranded virus belonging to family Bunyaviridae. WHO (1982) and Frederick *et al.* (1999). After the appearance of RVF disease in Egypt, Veterinary Serum and Vaccine Research Institute, Cairo, Egypt, succeeded in preparing safe and potent inactivated vaccine (EL Nimr, 1980). The reappearance of RVF disease in 1993 (EL Gabery *et al.,* 1994) increased the demand

to develop potent and less expensive vaccine. The inactivated vaccine was expensive and of short period of protection, so, Smithburn (1949) succeeded to produce an attenuated RVF vaccine which can protect the non pregnant sheep for a period of several years and less expensive. The reoccurrence of RVF disease in Egypt in mild form infection in 1993 encouraged the general organization for veterinary services to import and use the live attenuated vaccine to protect farm animals. The RVF Department produced local lyophilized attenuated Smithburn RVF vaccine with peptone and sucrose as stabilizer (Ibrahim 1996) and Elian *et al.* (1997).

The aim of this work is to develop this vaccine by using another stabilizer (skimmed milk) to improve the quality of the vaccine for a long time without great loss in its titre, to make the process of lyophilization more easier and also giving the disc of the lyophilized vaccine much better physical appearance.

MATERIALS AND METHODS

1- Animals

a. Hamsters

Four Syrian Golden hamsters of 6 - 8 weeks old were used for the Safety test.

b. Sheep

Ten adult sheep were selected, then, sera were tested by (SNT) for the detection of specific antibodies against RVF. They were found to be free from RVF neutralizing antibodies. They were used for evaluation of the attenuated RVF vaccine and to study the immune response of the prepared vaccine.

2- Virus

a. Virulent RVF virus

Rift Valley Fever Zagazig Human Strain (ZH501) was used and its titre was 107.5 TCID50 / ml. It was kindly supplied by RVF Department, Serum and Vaccine Research Institute, Abbassia, Cairo. It was used for SNT for detection of specific antibodies.

b. Attenuated RVF

The Smithburn's neurotropic strain (Smithburn, 1949) had a titre of 108 TCID50 / ml. It was used for the preparation of the attenuated RVF vaccine.

3- Cell culture

Monolayer BHK cell line was used for the production of attenuated vaccine titration and SNT according to EL Nimr (1980).

4- Stabilizer

Different concentrations of skimmed milk (10%, 15% and 20%) were added to the titrated virus with an equal volume.

5- Evaluation of the attenuated RVF vaccine

According to the protocol of WHO (1973).

a. Sterility test

The prepared vaccine was tested for its sterility, and it was found free from any bacterial, fungal mycoplasma contamination.

b. Safety test

1. The reconstituted vaccine in normal saline was inoculated into 2 hamsters by I/P route with 5 field dose ($5x10^4$TCID), and 2 hamsters were left non-inoculated as control. The hamsters were observed for 21 days for any symptoms or deaths.
2. The reconstituted prepared vaccine also was inoculated into 2 sheep I/V with 25 field dose of the vaccine and another 2 sheep were inoculated S/C with 25 field dose of the vaccine and were examined for a period of 21 days for any reaction or signs of clinical disease of RVF infection.

a. Potency test

1- Titration in T.C.

The prepared vaccine was titrated in tissue culture and its titre should not be less than 10^6 $TCID_{50}$ / ml.

2- *Seroconversion*

Serum neutralization test was done to measure the titre of the neutralizing antibodies in sera from sheep inoculated 1 field dose s/c of the prepared vaccine after 28 days of inoculation and the titre was not less than 1/40 according to the WHO Technical Report Series No. 323,1966 and No. 530, 1973.

6- Samples

Samples of the attenuated RVF vaccine with different concentrations of the stabilizer (skimmed milk solution) were kept at various temperatures according to the following design:

a-At room temperature, the vaccine was kept and the titre was measured daily for seven days.

b- At (4°C), the vaccine was kept and the titration was carried out every 3 months for one year.

c- At (−20C°) the vaccine was kept and the titration was done every 3 months for one year.

7- Vaccination of sheep

Six adult susceptible sheep were divided in to 2 groups as follows:

Group1. 4 sheep were vaccinated S/C with 1ml (field dose) of the Attenuated RVF vaccine.

N.B. These 4 sheep inoculated with the field dose were kept for following up the immune response to the vaccine.

Group2. 2 sheep were left as test control.

8- Serological test

The sera of sheep after vaccination were tested by SNT (Walker, 1970) for detection of antibodies against RVF virus at different intervals.

Table 1. Titration of attenuated RVF virus vaccine before and after lyophilization.

Samples	Titre $\log_{10}$ $TCID_{50}$/ml	
	Before lyophilization	After lyophilization
1- Vaccine fluid without stabilizer	8	N.D
2- Vaccine fluid with 10% skimmed milk	8	7.6
3- Vaccine fluid with 15% skimmed milk	8	7.6
4- Vaccine fluid with 20% skimmed milk	8	7.6

N.D = not done

Table 2. Evaluation of attenuated RVF vaccine with 10% skimmed milk.

Vaccine	Sterility	Safety									Potency	
		I/p inoculation of hamster			In sheep						In T.C cell	Sera Of Sheep After 28 days
					S/C			I/V			Log_{10}	Mean of
		1*	2*	3*	1*	2*	3*	1*	2*	3*		
Attenuated RVF Vaccine With 10% skimmed milk	Free from Any Fungal, Bacterial And mycoplasma Contaminants	2	2	0	2	2	2	2	2	0	7.6	80

1* - number of animals.

2* - alive animals. 3* - dead animals.

Table 3. Thermostability of the prepared lyophilized attenuated RVF virus vaccine at room temperature.

Time	Log_{10} $TCID_{50}$ /ml	Log_{10} reduction
0 Time	7.6	0.0
1st Day	7.3	0.3
2nd Day	6.3	1.3
3rd Day	5.6	2.0
4th Day	4.6	3.0
5th Day	3.3	4.3
6th Day	2.0	5.6
7th Day	1.6	6.0

Table 4. Thermostability of the prepared attenuated RVF virus vaccine at + 4 °C and – 20 °C.

Time	Log_{10} $TCID_{50}$/ml At + 4 °C	Log_{10} Reduction	Log_{10} $TCID_{50}$/ml At - 20 °C	Log_{10} Reduction
0 Time	7.6	0.0	7.6	0.0
1st month	7.6	0.0	7.6	0.0
3rd month	6.6	1.0	7.6	0.0
6th month	6.0	1.6	7.6	0.0
9th month	5.3	2.3	7.6	0.0
12th month	4.6	3.0	6.3	1.3

Table 5. Results of neutralizing antibody index (NI) in sera of sheep vaccinated with the prepared attenuated RVF virus vaccine with 10% skimmed milk as stabilizer.

Animals N0.	Treatment	Neutralizing index									
		Before Vaccination	After vaccination								
			1st Week	2nd Week	3rd Week	4th Week	2nd Month	3rd Month	4th Month	5th Month	6th Month
1	Attenuated RVF vaccine with skimmed milk	0.4	1.7	2.0	3.0	3.1	3.3	3.4	3.4	3.4	3.5
2		0.4	1.4	1.7	2.4	3.0	3.1	3.4	3.4	3.0	3.0
3		0.4	1.7	2.4	3.0	3.2	3.3	3.7	3.4	3.4	3.2
4		0.7	1.4	2.0	2.4	3.0	3.1	3.3	3.0	2.7	3.0
	Mean	0.47	1.55	2.03	2.7	3.08	3.2	3.45	3.3	3.13	3.18
1	Control non vaccinated animals	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4
2		0.7	0.7	0.7	0.7	0.7	0.7	0.7	0.7	0.7	0.7
	Mean	0.55	0.55	0.55	0.55	0.55	0.55	0.55	0.55	0.55	0.55

RESULTS AND DISCUSSION

Rift Valley Fever(RVF) disease is an economically important viral disease of animals. So, it is very important to control it by several methods, one of these methods is the vaccination of animals by using either inactivated or attenuated RVF vaccine. It is very important to study how to deal with RFV virus in the laboratories, especially, which produce huge quantities of vaccines or antigens. The reappearance of RVF disease in Egypt in 1993 (Weekly Epidemiological Record, 1994), encouraged to use attenuated RVF vaccine.

The purpose of the present work was to measure the stability and to improve the physical appearance of the lyophilized attenuated RVF vaccine using skimmed milk. The stock seed virus has an original titre of 10^8 $TCID_{50}$ / ml, after passing the virus in BHK cells and collecting the harvest. After addition of 10%, 15% and 20% skimmed milk as stabilizer to the virus, the titre was not changed and remained as 10^8 $TCID_{50}$ / ml. These products were subjected to lyophilization and the titre was reduced to $10^{7.6}$ $TCID_{50}$ / ml for all samples as shown in Table 1. These agreed with attenuated RVF vaccine (Elian *et al.* 1997) as they used peptone 5% or lactalbumen 5%. There was no difference between the different skimmed milk concentrations except in the physical appearance, (15% and 20%) had no good physical appearance. So we selected 10% skimmed milk attenuated RVF vaccine to complete this study. The final product was subjected to evaluation as recommended by WHO Technical Report Series No. 323 (1996) and WHO Technical Report Series No. 530 (1973). The results of this investigation revealed that the vaccine was sterile having no bacterial, fungal or mycoplasma conminants as tested by the standard techniques (Table 2).

Concerning the safety test, it was conducted in susceptible hamsters and the results showed no symptoms or deaths following inoculation. In sheep the results showed no clinical manifestation as shown in Table 2.

Results of thermo stability of attenuated RVF vaccine using 10% skimmed milk as stabilizer at room temperature presented in Table 3, revealed that the vaccine was still viable and effective within 48 hours with a titre of $10^{6.3}$ $TCID_{50}$ / ml. Then, the virus titre decreased until the 7^{th} day being $10^{1.6}$ $TCID_{50}$ / ml. This result agreed with Taradi (1997) who found that the vaccine had lost its infectivity titre within 7 days and the vaccine was not suitable for use.

Concerning results of thermo stability of lyophilized vaccine at +4°C represented in Table 4, revealed that the vaccine showed gradual decrease up till reaching $10^{4.6}$ $TCID_{50}$ / ml after 12 months, but, it was valid and effective for animal vaccination after 6 months at the same degree of temperature (+ 4°C) where, its titre was 10^{6} $TCID_{50}$ / ml, while, the present attenuated RVF vaccine with (5% lacto albumen & 2.5% sucrose) can be preserved at + 4°C for only 5 months as said by Taradi (1997).

Concerning results of thermo stability of the vaccine at more lower temperature (-20°C), it was revealed that there was no reduction in the titre of the attenuated RVF vaccine up till the 9th month with a titre of $10^{7.6}$ $TCID_{50}$ / ml, and also, the vaccine was valid and effective being $10^{6.3}$ $TCID_{50}$ / ml for 12 months which is the log reduction =1.3. (The end of the experiment as shown in Table 4.

According to the results of seroconversion in sheep, the neutralizing antibodies against RVF was shown in Table 5. It was found that the antibodies were in the protective level from the 1st week with an average of NI (1.55) [following (Pini, 1973) who said that the protective level being with 1.5]. The level of antibodies reached its peak at the 3rd month post-vaccination with an average of NI (3.45) and was still high with an average of NI (3.18) up till the duration of experiment which was 6 months.

From this study, it was found that the 10% skimmed milk is the best stabilizer of choice. Also, we can store this attenuated RVF vaccine at –20 °C without loss in its titre till 9 months, while, at room temperature it is suitable for preservation of the vaccine for 48 hours and 4 °C is suitable for 6 months.

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محاولات لاستخدام اللبن المنزوع الدسم كمادة توازن للقاح حمى الوادى المتصدع المستضعف

للى صبحى سلامة ، تيمور نصيف مرقص ، جيهان كمال محمد ،
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فى هذة الدراسة تم تحضير و إستخدام اللقاح المستضعف لحمى الوادى المتصدع بعد إضافة اللبن المنزوع الدسم كمادة توازن و ذلك بتركيزات ١٠% و ١٥% و ٢٠% و بعد التجفيد وجدنا أنة ليس هناك فرق فى كفاءة التركيزات المختلفة و أفضلها فى الخواص الطبيعية هو التركيز ١٠ % و بإجراء إختبارات التقييم و المعايرة للقاح و جد أن عياريتة كانت $10^{7.6}$ $TCID_{50}$ / ml و بإجراء أختبار السلامة فى الجربوع و الأغنام وجدنا اللقاح آمنا. وأيضا إختبار كفاءة اللقاح فى الخلايا كان $10^{7.6}$ $TCID_{50}$ / ml و تم معايرة اللقاح الجديد فى درجـــات الحفظ المخـــتلفة و كانت النتائـــج كالأتى عند ٢٥ م (٧,٦) فى اليوم الأول ووصلت (١,٦) عند اليوم السابع بينما فى درجة حرارة ٢٠ م (٧,٦) فى الشـــــهر الأول ووصـــل عند (٤,٦) فى الشهر الثانى عشر ثم فى درجة حرارة –٢٠ م كانت القوة العيارية (٦,٣) حتى الشهر الثانى عشر من الدراسة. وبالتقييم للمستوى المناعى للقاح كان مرتفعاً حتىالشهر السادس و ذلك بمستوى (٣,١٨) فى إختبار التعادل المصلى . و من هذة الدراسة لاحظنا أن اللبن المنزوع الدسم أفضل فى إمكانية تخزينة عند ٢٥ م لمدة ٤٨ ساعة و ٦ شهور عند ٤ م وفى نفس الوقت ممكن تخزينة عند –٢٠ م بدون فقد قوتة حتى ٩ شهور.