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PRODUCTION AND EVALUATION OF ANTI RIFT VALLEY FEVER (RVF) IgG CONJUGATED WITH FLUORESCEIN

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Abstract

Three susceptible sheep were inoculated with RVF antigen mixed with incomplete and complete Freund's adjuvant and without adjuvant in three successive doses. The hyperimmune sera collected from the sheep were tested against standard reference RVF antigen for the presence of specific RVF antibodies using serum neutralization test (SNT), agar gel precipitation test (AGPT) and indirect fluorescent assay (IFA). Separation of RVF immunoglobulins were done using affinity chromatography (activated sepharose 4B) followed by conjugation of RVF IgG with fluorescein isothiocyanate at pH 9.6. The conjugated RVF antibodies were titrated against reference RVF antigen and the titre reached (1:128). The working dilution was used for evaluation of different batches of attenuated and inactivated RVF vaccines in comparison with IFA test using anti-sheep Fitic, the results of both assays proved to be similar.

INTRODUCTION

Rift Valley Fever (RVF) is an acute infectious disease causing a high mortality rate in farm animals and human beings (Daubney *et al.*, 1931). The disease which was recorded for the first time in Egypt in 1978 caused high economic losses in young calves, lambs and death in human beings (Meegan and Moussa, 1979). The recent reoccurrence of RVF in 1993 (Arthur *et al.* 1993) revealed the need for a more practical and specific tool for rapid diagnosis of the disease to facilitate the application of rapid control measures.

The aim of this study was to produce RVF IgG antibodies conjugated with fluorescein isothiocyanate in a specific good titre, high amount and cheaper in price for rapid detection of RVF infection, as well as, evaluation of different types of RVF vaccines.

MATERIALS AND METHODS

Materials

1. Virus (ZHMC21): Rift Valley Fever virus used in this study (**Taha, 1982**) was kindly supplied by RVF Dept., Veterinary Serum and Vaccine Research Institute (VSVRI).

2. RVF antigen and antisera: The purified reference RVF antigen and antisera were obtained from RVF Dept., VSVRI.

3. Sheep: Three susceptible sheep tested against RVF virus were used for preparation of RVF hyperimmune sera.

4. Fluorescein isothiocyanate: (Sigma Co., USA).

5. Dialysis bag: (Sigma Co., USA).

6. Sepharose 4B cyanobromide: (Sigma Co., USA).

7. Anti-sheep Fitic: (Sigma Co., USA).

Methods

1. Preparation of RVF hyperimmune sera

Each sheep was inoculated intramuscularly (I/M) with 1ml of RVF antigen mixed with 1ml of complete Freund's adjuvant. A booster dose was given 2 weeks later consisting of equal amounts of antigen and incomplete Freund's adjuvant. Two weeks later, a final dose of antigen alone was given via same route. After a period of two weeks rest, serum samples were collected and subjected to evaluation.

2. Evaluation of sheep sera

a. Agar gel precipitation test (AGPT)

It was applied according to El-Nimr (1980) using reference RVF antigen and antisera as controls against the tested sheep sera.

b. Serum neutralization test (SNT)

The technique was described by Walker *et al.* (1970) where constant 100 $TCID_{50}$ of reference RVF virus was tested against two fold dilutions of the prepared sheep serum. The neutralizing indices were calculated according to Reed and Muench (1938).

c. Immunofluorescent assay (IFA)

The technique was described by Elian *et al.* (1996) where, 96 well tissue culture plates containing BHK cells were infected with 100 $TCID_{50}$ of reference RVF virus. The appearance of cytopathic effect on the BHK cells occurred 48 hours post-inoculation of the virus, then, the plates were fixed with absolute ethanol. Two fold dilutions from the tested sheep sera were added to the plate which was incubated at 37°C for 45 minutes, washing with phosphate buffer saline (PBS) of pH 7.2 for 3 times. Anti-sheep filtic was used for the identification of the tested sheep sera.

d. Estimation of protein content in sheep sera

The level of protein in sheep sera was measured by Biuret method according to Canon *et al.* (1974). The results were expressed as gm/100ml using spectrophotometer at 540 nm wavelength.

3. Separation and purification of anti-RVF IgG by affinity chromatography

Anti-RVF IgG was separated and purified by batchwise procedure on cyanobromide activated sepharose 4B according to methods described by Anderson *et al.* (1975).

4. Conjugation of RVF IgG antibodies with fluorescein

- 2.5 ml of 0.2 M disodium hydrogen phosphate solution (Na_2HPO_4) were added to 10ml serum fraction containing approximately 1% of estimated serum protein which was mixed with a magnetic stirrer.

- 2.5 mg of pure fluorescein isothiocyanate (Sigma, USA) were dissolved in 2.5 ml of 0.2 M Na_2HPO_4 solution with addition of 2.5 ml bidistilled water. This solution was added to the protein solution slowly under constant stirring which took about 15 minutes with continous adjustment of pH to 9 with 0.1 M carbonate bicarbonate buffer. Then, PBS of 7.2 pH was added to make the total volume 20ml. The mixture was kept overnight at 4°C without stirring, then, dialysis against PBS 7.2 pH for 3 days at 4°C with changing the PBS twice daily to remove undesired fluorescein. This technique was described by Nowotony (1979).

5. Evaluation of conjugated anti-RVF IgG with fluorescein

a. A drop of conjugated material was put on a filter paper strip, dried and the fluorescence was observed under the fluorescent microscope (Nowotony, 1979).

b. Conjugated RVF IgG with fluorescein was titrated against 100 $TCID_{50}$ of reference RVF antigen to detect the most suitable dilution of conjugated material to prepare working solution. The results obtained were compared with a control IFA using reference antigen, antisera and anti-sheep Fitic.

6. Field application for the conjugated material

a. Evaluation of attenuated RVF vaccine (Identity and Titration)

Direct FAT was done in 96 well tissue culture containing BHK cells infected with different dilutions of the tested batches of the attenuated RVF vaccines as described by Elian *et al.* (1996) and Wassel *et al.* (1997). Results are given in Table 3.

b. Evaluation of binary inactivated alum gel RVF (Identity test)

Different batches of inactivated RVF vaccines were tested in this experiment as described by Wassel *et al.* (1997).

RESULTS AND DISCUSSION

The immune response of sheep inoculated with RVF antigen reached > 3 SNT index when measured by SNT (Table 1), and RVF antibodies were identified also by IFA (Photo 1) and AGPT (Table 1). The results indicated that the sheep produced specific antibodies against RVF inoculated antigen. This comes in agreement with Eman (1995) and Wassel *et al.* (1997).

The amount of protein present in the serum of immunized sheep reached 3.76 ug/0.1 ml which was suitable quantity to be used in the conjugation of antibodies with fluorescein (Karl and Norman, 1969) where they used 2ug/0.1 ml protein for conjugation.

The use of incomplete and complete Freund's adjuvants enhanced the immune response of inoculated sheep with RVF antigen to obtain high titre of antibodies against RVF (El-Nimr, 1980).

The use of saturated ammonium sulphate and cyanobromide activated sepharose 4B in the affinity chromatography played an important role in the separation and purifi-

cation of RVF IgG antibodies with high titre (Anderson *et al.*, 1975) which was used for the conjugation with fluorescein.

The titre of conjugated RVF IgG antibodies reached 1:128 (Table 2) when reacted with RVF antigen. This became the working dilution used for evaluation of RVF vaccine (identity) (Table 2 and Photo 2). The titre of attenuated RVF vaccine using the prepared conjugated anti-RVF IgG with fluorescein reached 2 log $TCID_{50}$/dose. This result came in agreement with those of Elian *et al.* (1996) when they used IFA.

From all the above mentioned, we can say that we can prepare anti-RVF IgG conjugated with fluorescein for the commercial scale of low price with good titre and could be used for diagnosis of RVF infection and for evaluation of attenuated RVF vaccine (titration and identity test) and for inactivated one (identity test) instead of using IFA for detection of RVF antigen which is expensive.

Table 1. Evaluation of prepared sheep RVF antibodies before conjugation with fluorescin.

No. of sheep	Susceptibility of sheep against RVFV Pre-vaccination	Indirect fluorescent assay (IFA) using specific anti-sheep Fitic 6 weeks post inoculation with RVFV antigen		Serum neutralization test		Agar gel precipitation test (AGPT)		Purity test	Identity test	Amount of serum protein per 0.1 ml
		Control positive serum	Tested serum	Control positive serum	Tested serum	Control positive serum	Tested serum			
1	0	+	+	> 3	> 3	+	+	Ster.	RVF	4.2 ug/0.1
2	0	+	+	> 3	> 3	+	+	Ster.	RVF	3.1 ug/0.1
3	0	+	+	> 3	> 3	+	+	Ster.	RVF	4.0 ug/0.1
Mean	0	+	+	> 3	> 3	+	+			3.76 ug/0.1

+ : Presence of antibodies against RVF.
Control positive : using specific reference RVF antibodies and RVF antigen.
Log SNI : log serum neutralizing index.
Ster. : Free from contaminants (virus, bacteria and mycoplasma).

Table 2. Titration of conjugated anti-RVF IgG with fluorescein using reference RVF antigen.

Dilution of conjugate ⇨ / Type of antigen ⇩	Anti-RVF IgG conjugated with fluorescein									
	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256	1/512	1/1024
Reference RVF antigen	+	+	+	+	+	+	+	-	-	-
Negative control	-	-	-	-	-	-	-	-	-	-
Control negative cells	-	-	-	-	-	-	-	-	-	-

+ : Specific antigen antibody reaction of RVF IgG.
- : No antigen antibody reaction.

Table 3. Evaluation of RVF vaccines (Identity test) using prepared antisheep IgG conjugated with fluorescein.

Type of vaccine	Direct fluorescent assay (using prepared material)		Control indirect immunofluorescent assay using antisheep fitic	
	Identity	Titre	Identity	Titre
1. Attenuated RVF vaccine :				
a. Batch No. 1/97	RVF	2.5 $\log_{10}$ $TCID_{50}$/dose	RVF	2.0 $\log_{10}$ $TCID_{50}$/dose
b. Batch No. 2/98	RVF	2.5 $\log_{10}$ $TCID_{50}$/dose	RVF	2.5 $\log_{10}$ $TCID_{50}$/dose
c. Batch No. 3/98	RVF	2.0 $\log_{10}$ $TCID_{50}$/dose	RVF	2.0 $\log_{10}$ $TCID_{50}$/dose
2. Inactivated RVF alum gel vaccine :				
a. Batch No. 154/99	RVF vaccine		RVF vaccine	
b. Batch No. 155/2000	RVF vaccine		RVF vaccine	
c. Batch No. 156/2000	RVF vaccine		RVF vaccine	
3. Reference control RVF antigen	RVF virus		RVF virus	
4. Negative control antigen	Negative		Negative	

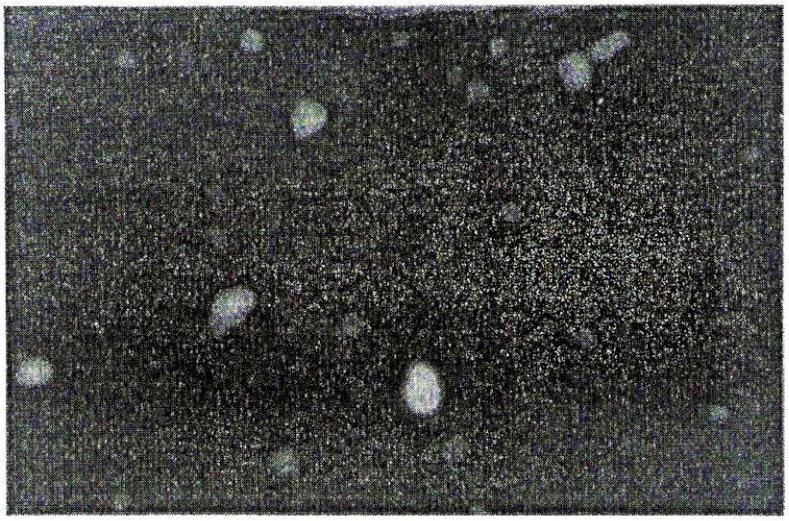

Photo 1. IFA x 140

RVF antigen detected by anti-sheep fitic.

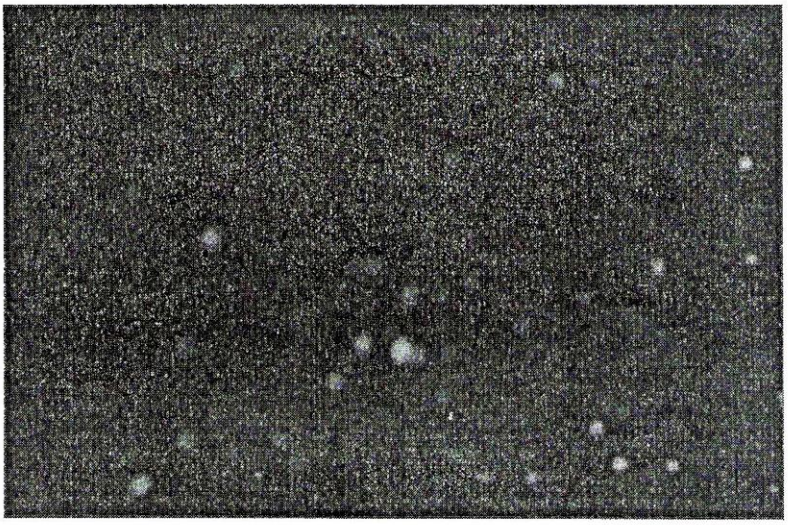

Photo 2. FA x 140

RVF.antigen detected by anti-RVF IgG conjugated with fluorecein.

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إنتاج وتقييم أجسام مناعية ضد فيروس حمى الوادى المتصدع واتحادها بالفلورسين

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تم حقن ٣ أغنام بأنتيجين حمى الوادى المتصدع المرجعى بثلاث جرعات باستخدام مادة الفروينداز الغير كامل والكامل وبدون مادة حاملة. تم تجميع المصل المحتوى على الأجسام المناعية العالية بعد شهرين من بداية الحقن وتم اختباره باستخدام أنتيجين حمى الوادى المتصدع المرجعى بتطبيق اختبار التعادل المصلى والترسيب فى الآجار واختبار الفلورسنت المشع الغير مباشر وكذلك قياس نسبة البروتين. تم فصل الأجسام المناعية ج من مصل الأغنام باستخدام السيفاروز النشط (فى عمود كروماتوجرافى المتشابه) واتحادها بمادة الفلورسين عند درجة حموضة ٩,٦ . وتم تنقية المادة المحضرة باستخدام أنابيب سليلوز للتنقية من الفلورسين الغير متحد بالأجسام المناعية ثم تقييم المادة المحضرة باستخدام أنتيجين حمى الوادى المتصدع المرجعى وأعطى قوة عيارية وصلت إلى ١:١٢٨ وتم استخدامها لمعايرة دفعات من لقاحات حمى الوادى المتصدع المستضعفة والمثبطة وتم مقارنتها باستخدام اختبار الفلورسنت الغير مباشر وقد وجد تطابق فى النتائج بينهما.