Assiut Vet. Med. J. Vol. 37 No. 73, April 1997.

Serum and Vaccine Research Institute,
Abbasia, Cairo, ARC.

RAPID EVALUATION OF LIVE ATTENUATED AND INACTIVATED RIFT VALLEY FEVER VACCINES IN-VITRO

(With 6 Tables, 2 Photo. and 2 Fig.)

By

M.S. WASSEL; K.A. ELIAN; F.F. ZAKI and ELHAM A. EL-IBIARY

(Received at 9/3/1997)

استخدام طرق معمليه سريعه لتققيم لقاح الرفت فالى الحى المستضعف والمثبط

محمد سعيد واصل ، خيرات عليان ، فريد زكى
إلهام الإبيارى

تم استخدام اختبار الفلورسنت الغير مباشر للتعارف ولمعايرة ١٠ دفعات من لقاح الرفت فالى الحى المستضعف بعد حقنها بتخفيفات مختلفه فى خلايا كلية الجربوع السورى وقد أعطى قراءة قوة عياريه وصلت إلى ٥ر٧ لو ١٠ بعد ٢٠ ساعه من الحقن. وذلك بالمقارنه بلقاح مرجع للرفت فالى الحى المستضعف السابق معايرته فى حيوانات حقليه ومعمليه. تم أيضاً استخدام أختبار الفلورسنت الغير مباشر للتعرف على لقاح الرفت فالى المثبط وذلك بصبغة كرات الجيل الحاملة لأنتجين الرفت فالى بعد تثبيته على شرائح زجاجيه بالأسبنون بعد تجفيفها عليها. تم عمل منحنى قياسى لبروتين مرجع بإستخدام طريقة البراوفورد بعد عمل تخفيفات مختلفه لأنتجين الرفت فالى المرجع والنقى باستخدام مقياس الطيف وصبغة الكوماسى الأزرق ثم معرفة كمية البروتين فى كل تخفيف. تم استخدام اختبار الاليزا متعدد الطبقات لتقييم لقاح الرفت فالى المثبط وذلك بعد إجراء اختبار لوحة الشطرنج، وتم إجراء الاختبار بعمل تبطين لأطباق الاليزا من الأجسام المناعيه المضاده للرفت فالى وبعد ٢٤ ساعة ثم استخدام التخفيفات المختلفه من انتجين الرفت فالى والمعروفه كمية البروتين بها بالميكروجرام مع تخفيفات من عدد ١١ دفعه من اللقاحات المختبره ولقاح الرفت فالى المرجع السابق معايرته فى حيوان الحقل وبعد قراءة الاليزا تم عمل منحنى قياسى (عيارى) من قراءة درجة كثافة الطيف وكمية البروتين فى الانتجين المرجع والذى تم استخدامه لمعرفة كمية البروتين فى اللقاحات المختبره وقد أعطت كمية بروتين وصلت ٥ ميكروجرام فى المللى (الجرعه) كذلك تم مقارنة درجة كثافة الطيف فى اللقاح المرجع مع اللقاح المختبر وكانت النسبه ١ : ١ وكانت نتائج درجة كثافة الطيف فى الدفعه المرجعيه بالجيل مساويه لدرجة كثافة الطيف لنفس الدفعه بدون جيل مما يعزى أن أختبار الاليزا يعطى درجة احلال للأنتجين من الجيل تصل إلى ١٠٠٪. ومما سبق يمكن القول بأنه باستخدام اختبار الفلورسنت

الغير مباشر واختبار الاليزا المزوج الطبقات مع اختبار السلامه والنقاوه والمقارنه بلقاح رفت فالى سبق معايرته فى حيوان التجارب ممكن أن يوفر الجهد والوقت وتكاليف المعايره.

SUMMARY

Indirect immunofluorescence assay (IFA) was used for detection, identification and titration live attenuated RVF (7.5 $\log_{10}$ $TCID_{50}$ / ml) as well as inactivated alum gel RVF. Bradford's method (1976) was used to determine the amount of ug / 0.1 ml of purified reference RVF antigen (1:2000 - 1:32000) dilutions. Double sandwich ELISA was used to compare the tested inactivated RVF vaccine with a reference one has passed animal potency testing as well as to determine the amount of protein (ug / dose) of the tested inactivated RVF vaccines by using different dilutions of purified RVF antigen of known protein concentration (ug / 0.1 ml) and then a standard curve was prepared by regression analysis of the purified RVF antigen dilution of known protein concentration against its ELISA optical density (O.D.) at A450 and the protein in concentration of the reference RVF vaccine was determined by interplotting its ELISA O.D. at A405 on the RVF standard curve which reached 5 ug/ dose of RVF vaccine. Also, the ELISA potency ratio was determined by comparing the O.D. of tested vaccine to that of reference inactivated RVF vaccine which has passed animal potency testing and the main value of the potency ratio reached 1.00.

Key Words: Rift Valley Fever-Vaccines-Evaluation.

INTRODUCTION

Rift Valley Fever (RVF) was first reported in Egypt in 1997 in a massive outbreak among animals with extensive involvement of humans (WHO, 1977, Al-Akkad, 1978 and Meegan, 1979).

The recent recurrence of RVF in 1993 (Arthur et al., 1993) revealed the need for a more practical and more efficient of quality control for both live attenuated RVF vaccine (Smithburn, 1949) and inactivated alum gel RVF vaccine (Barnad and Botha, 1977 and Eman, 1995).

The general requirement for live attenuated RVF vaccine according to WHO (1966 and 1973) and U.S. Code of Federal Regulation (9 CFR) (1987) depends on purity, safety, virus titre and virus identity tests by using indirect immuno-fluorescence assay (IFA) (Elian et al., 1996). As regard to inactivated RVF vaccine purity, identity and quality of the recovered antigen

through elution or separation by biological affinity or adsorption interaction, the amount of the isolated antigen protein (ug / dose) can be then identified, measured and screened with suitable antibodies immunological activity (Niklasson et al., 1983; Shiari et al., 1990 and Peters, 1993).

Sandwich ELISA have been used for measurement of viral proteins (Rose et al., 1986 and WHO, 1983).

An ideal vaccine would be one that could provide more than 90% efficacy within a few weeks of single administration of vaccine, a protection would be of long duration and of low cost, (Peters, 1993).

Our aim of work is to use a rapid, practical, efficient and of low cost method, to save time and to minimize the cost of evaluation of RVF vaccines.

MATERIALS and METHODS

MATERIALS:

1- Virus :

Rift Valley. Fever virus used in this work was designated as $ZHMC_{21}$ (Taha, 1982).

2- RVF antigen and antisera:

the purified reference RVF antigen and purified sheep anti-RVF and purified bovine anti-RVF immunoglobulin were kindly supplied from RVF Dept., Vet. serum and Vaccine Research Institute.

3- Rabbit antibovine IgG conjugated with peroxidase (Sigma Immunochemical Company).

4- Rabbit antibovine IgG conjugated with fluorescein (Sigma Immunochemical Company).

3. Cell Culture:

Monolayer BHK cell cultures were grown and maintained as described by El-Nimr (1980).

4. Vaccines:

Ten batches of live attenuated RVF vaccines and 11 batches of binary inactivated alum gel RVF vaccines, prepared at RVF Department, Vet. Serum and Vaccine Research Institute, Abbasia, Cairo, were used in this study.

Methods:

The reference RVF vaccines were evaluated according to the following:

1. ***Purity test*** was performed in accordance with U.S. Code of Federal Regulation 9 CFR (1987) testing 113.26, 113.27, 113.30 and 113.55.
2. ***Safety test:***
   - ***a. Live attenuated RVF vaccine:***
     According to WHO (1966, 1973), Golden Hamsters, sheep and cattle were used as described in 9 CFR (1987) testing 113.41.
   - ***b. Binary inactivated alum gel RVF vaccine:***
     20 Baby mice and 3 lambs of 10 days old used as described by Eman (1995).
3. ***Virus Identity:***
   The virus identity tests were conducted on RVF vaccine according to 9 CFR (1987) testing 113.135 and WHO (1966) using:

Idirect Immunofluorescent assay (IFA):

a. Live attenuated RVF vaccine:

IFA was done using 96 well tissue culture plates containing BHK cell infected with different dilutions of live attenuated RVF vaccines from different batches and compared with the reference one. After 20 hours, the plates were fixed and RVF virus was titrated and identified as described by Elian et al. (1996) and Wassel et al. (1996).

b. IFA for identity test in case of Binary inactivated alum gel RVF vaccine:

It was carried out on a slide where a drop of final products of inactivated RVF vaccine smeared, fixed using dry air and acetone then RVF antigen was identified using IFA as described by Johnson et al. (1981).

Potency Test :

I. In-vivo potency test (reference):

1. Live attenuated RVF vaccine:

It was carried out in 4 susceptible sheep inoculated with 1 ml S/C as described in WHO (1966) and (1973)and inoculation was repeated after 2 weeks.

2. Binary inactivated alum gel RVF vaccine:

It was carried out in 60 adult mice and the ED_{50} was calcuated as described by Reed and Muench (1938) and 4 susceptible sheep inoculated with 1 ml S/C of inativated RVF vaccine as described by Eman (1995).

II. In-vitro potency assays:

A. Measurement the amont of microgram (ug) of protein of purified reference RVF antigen:

Quantitation of ug of protein of the RVF antigen was done by measuring the absorbance of different dilution of RVF antigen from (1:200 to

1:32000) in 0.15 M NaCl and 0.1%. coomassie brilliant blue stain (G.250 and measured by spectrophotometer at A595 and then by plotting the recorded optical density of RVF antigen on the prepared standard BSA curve from 0.5 ug - 9 ug / 0.1 ml as described by Bradford (1976).

B. Sandwich ELISA for measurement of Droteins (Rose et al., 1986):

Chess board titration to find the working dilutions of the reagents:

1. The purified sheep anti-RVF diluted in coating buffer to give 10, 2 and 0.5 ug of protein in 0.1 ml as in Bradford (1976). 100 ul of each dilution was added to 3 vertical columns of wells in a microplate, then kept in a humid chamber for 18 hours at 4°C and then washed with PBS-T in the usual way.
2. 100 ul of the strongly positive purified RVF antigen solution (1:200) was added to one horizontal row well and add the weakly positive (1:16000) of purified RVF antigen and negative one to other horizontal row. Then the plate was incubated for 2 hours at room temperature and washed with PBS-T in the usual way.
3. The purified bovine anti-RVF virus was diluted into three dilution 1:400, 1:800 and 1:600, then 100 ul of each dilution was added to one vertical row of the plate coated with each level of antigen. The plate then was incubated for 1 hour at room temperature and then washed in the usualy way.
4. Antibovine conjugated with peroxidase in 1:3000 dilution in PBS (pH 7.4) was added to each well, left for 1 hour at 37°C. The plate then was washed with PBS-T in the usual way and then followed by addition of 100 ul of substrate solution (5-amino salicylic acid) and H_2O_2. The developed color had been read in an ELISA reader (Rose et al., 1986) and working dilution of reagents could be found.

C. Sandwich ELISA for measurement of the amount of microgram of RVF vieral Drotein per dose:

It was done according to the (USDA, National Vet. Labs and WHO, 1983).

a. Microtiter plates (96 well) were used in the double antigen sandwich ELISA for RVF antigen protein where an appropiate amount of working reagent were determined as described before.
b. The purified sheep antisera against RVF virus were diluted (2 ug / 0.1 ml) in coating buffer (pH 9.6). The plate was incubated at 4°C overnight, followed by washing with PBST in usual way and the plate was post coated for 20-30 minutes at room temperature with 150 ul of 2% BSA and the plate was washed again 3 times with PBST.

c. Serial 2 fold dilutions of purified RVF antigen from 1:200 to 1:32000 (the protein contation "ug / 0.1 ml) of each dilution of the purified RVF antigen was known as mentioned before from 0.5 ug to 6.2 ug / 0.1 ml).
d. 1:10 dilution of the tested RVF vaccine as well as the reference inactivated alum gel RVF vaccine were added to duplicated well 100 ul / well of the antibody coated plate which incubated at 37°C for 2 hours then washed 3 times with PBST. The purified bovine anti-RVF virus was diluted to 1:400, then added to each well (100 ul / well) and the plate was incubated at 37°C for 1 hour, then washed 3 times with PBST.
e. Antibovine peroxidase IgG was added in dilution of 1:3000 in each well. The plate then washed with PBST in the usual way and then followed with addition of 100 ul of (5-amino salicylic acid) and H_2O_2. The developed colour had been raed in ELISA reader at A405.
f. A standard curve was prepared by regession analysis of the purified RVF antigen dilution of known protein concentration against the ELISA optical density (O.D.) at A405.
g. The protein concentration of the tested vaccine as well as the reference RVF vaccine was determined by interplotting the recorded ELISA optical density at A405 on the purified RVF virus standard curve.

RESULTS

The live attenuated RVF virus vaccines were detected and titrated on BHK cell line after 20 hours following infection and the titre reached 7.5 $\log_{10}$ $TCID_{50}$ / ml by using IFA as photo (1) and Table (1). The neutralizing titre reached 64 and the tested live reference RVF vaccine was safe in sheep and golden hamster and cattle (Table 1).

As regard to evaluation of the reference binary inactivated alum gel RVF vaccines, the vaccine was sterile, safe in baby mice and lambs and the vaccine was potent following challenge with pantropic RVF virus as the ED_{50} in mice reached 0.014 / ml and the neutralizing indices reached 3.1 after 4 weeks and reached 2.00 after 6 months post vaccination (Table 2) and vaccine was identified by using IFA as RVF antigen (Photo 2).

Using the standard curve of Bradford methods (Table 3 and Fig. 1), the protein concentration of the purified RVF antigen was determined from 0.5 ug - 6.2 ug / 0.1 ml at (1:200 - 1:16000) dilution of RVF antigen (Table 4).

The in-vitro double sandwich ELISA potency ratio as indicated when comparing the reference binary inactivated alum gel RVF vaccine with the

tested batches of inactivated alum gel RVF, the ratio reached 1.00 as in Table (6).

Also, the result of measurement of the amount of microgram of RVF vaccinal viral protein per dose compared with reference purified RVF viral protein (Table 5) ranged from 0.4 - 0.6 ug / 0.1 ml with the average of 0.5 ug / 0.1 ml or 5 ug / dose as in Fig. (2) of tested and reference binary inactivated alum gel vaccine (Table 6).

As in Table (6), the ELISA O.D. of the reference inactivated RVF vaccine with gel reached 0.462 and the ELISA O.D. of the inactivated RVF vaccine without alum gel reached 0.462 at A405.

DISCUSSION

Using IFA technique to identify and titrate live attenuated RVF vaccine after 20 hours post infection where this rapid result 7.5 $\log_{10}$ $TCID_{50}$ / ml agreed with that finding in testing 113.135 9 CFR (1987) and WHO (1966, 1973) when compared with the reference one have been passed animal potency testing.

The result obtained for evaluation of reference inactivated RVF vaccine as it was identified by IFA and of good immunogenic response (NI reached 3.1) and safe agreed with those obtained by Eman (1995).

Using double sandwich ELISA to detect RVF to detect RVF virus antigen agreed with Niklasson et al. (1983); WHO (1983) and Rose et al. (1986).

The result of in-vitro ELISA potency ratio (1.00) when comparing the testing inactivated RVF vaccine with the reference one came with agreement with those of USDA National Vet. Laboratories. The amount of microgram of RVF viral protein per dose (5 ug / dose) agreed with those reported by Shiari et al. (1990) for evaluation of FMD where it reached 2-4 ug / dose.

As the ELISA O.D. (0.462) of RVF vaccine with equal the O.D. of the same lot of RVF vaccine without gel (ELISA O.D. 0.46), we used the double sandwich ELISA to detect RVF antigen in inactivated alum gel vaccine by using antigen antibody interactions (Peter, 1993) gave good result instead of biological affinity, as the later leave 40% - 70% of the antigen in alum gel after elution (Shiar et al., 1990).

We can say by using IFA to detect, identify and titrate live attenuated RVF vaccine in BHK cell line and using in-vitro ELISA potency ratio when one has passed host animal potency testing besides studying the safety tests

values could be used with success for rapid and low cost of evaluation of RVF vaccine.

REFERENCES

Al-Akkad, A.M. (1978): Rift Valley Fever outbreak in Egypt (October - December 1977). J. Egypt. Publ. Health Assoc., 55: 123 -128.

Arthur, R.R.; El-Sharkawy, M.S.; Cope, S.E.; Botros, B.A.; Oun, S.; Morill, J.C; Shope, R.E.; Hibbs, R.G.; Darwich, M.A.; Imam, I.Z.E. (1993): Recurrence of Rift valley fever in Egypt. Lancet, 342: 1149 - 1150.

Barnad, D.H.J. and Botha, M.J. (1977): An inactivated Rift valley fever vaccine. J. South Afr. Vet. Assoc., 48; 45-48.

Bradford, M.M. (1976): A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein dye binding. Analytical Chemistry, 72: 248 - 254, Academic Press, Inc.

Elian, K.A.; Wassel, M.S.; Mohamed, Gihan, K.M. and El-Debegy, Aida, I. (1996): Serological studies following vaccination with attenuated Rift valley fever (RVF) vaccine in Egypt. 4th Sci. Cong. Proc., April 3-6 (1996), Vet. Med. J., Giza, 44 (2): 409-414.

El-Nimr, M.M. (1980): Studies on inactivated vaccine against RVF. Ph.D. Thesis, Microbiology, Fac. Vet. Med., Assiut Univ., Egypt.

Eman, E.S. (1995): Studies on RVF vaccines inactivated with binary. Ph.D. Thesis, Microbiology, Fac. Vet. Med., Cairo Univ., Egypt.

Johnson, K.M.; Elliott, L.H. and Heyman, D.L. (1981): Preparation of polyvalent viral immunofluorescent intracellular antigens and use in human sero-surveys. J. Clin. Microbiol., 14: 529.

Meegan, J.M. (1979): Rift valley fever epizootic inEgypt (1977 - 1978). Description of the epizootic and virological studies. Trans. R. Trop. Med. Hyg., 73: 618-623.

Niklasson, B.; Grandien, M.; Peters, C.J. and Gardan, T.P. (1983): Detection of Rift valley fever virus antigen by enzyme linked immunosorbent assay. J. Clin. Microbiol., 17: 1026-1031.

Peters, R.A. (1993): Identification of suitable antigens and antigenic sites for subunit or vectored vaccines. Vaccines for Vet. Applications, First Publish 1993, pp. 32-59, Butterworth, Heineman Ltd., London.

Reed, L.J. and Muench, H.A. (1938): A simple methods for estimating 50% end points. N.Am. J. Hyg., 27: 493-497.

Rose, V.R.; Friedman, H. and Fahey, J.L. (1986): Enzyme linked immunosorbent assay. Manual of Clin. Lab. Immunol., pp. 107-109, American Society for Microbiology, Washington, D.C.

Shiari, J.; Chatchawanchonnteera, A.; Sinsuwongwat, W.; Makarasen, P. and Sugimure, T. (1990): Estimation of 1405 particles in foot and mouth disease virus (FMDV) vaccines by using the computer analysis system. Jap. J. Vet. Sci., 52 (3): 621-630.

Smithburn, K.C. (1949): Rift valley fever: The neurotropic adaptation of virus and experimental use of this modified virus as vaccine. British J. Exper. Pathol., 30: 1-6.

Taha, M.M. (1982): Studies on inactivated vaccine against RVFV. Ph. D. Thesis, Microbiology, Fac. Vet. Med., Cairo Univ.

United state code of federal Regulation (1987): Animal and animal products 9 (1987): Published by Office of Federal Register Naster National Archives and Records Administration.

Wassel, M.S.; Susan El-Mahdy; Elham, A. El-Ibiary and N. Ali, A.M. (1996): Rapid detection and titration of Marek's disease virus (MDV) vaccine on CER cell line. Assiut Vet. J., 35 (70): 148-156.

WHO Technical Report No. 323 (1966).

WHO Technical No. 530 (1973).

WHO (19770: Rift vally fever, World Health Organization, Weekly Epidemiological Rec., 50-401.

WHO (1983): Rift valley fever, Laboratory diagnostic procedure workshop on RVF held at Aristotolian University of Thessalonik, Greece, 6-10 June, 1983, Sponsored by WHO/UNDP.

Table (1) : Evaluation of the live attenuated RVF vaccine.

Purity	Identity test using IFA	Titre log_{10} $TCID_{50}$/ml	Safety			Animal Inoculation Mean serum titre following * vaccination with live RVF vaccine						
			Hamster	Sheep	Cattle	0	1 st Week	2 nd Week	Second Vaccination	4 th Week	Challenge	6 th Week
Sterile	RVF virus	7.5 log_{10} $TCID_{50}$/ml	Safe			0	2	16		64		64

* Titre expressed as reciprocal of serum dilution.
› 40 is the protective the level after 28 days.

Table (2): Evaluation of the reference inactivated binary alum gel RVF vaccine in sheep.

Purity	Identity IFA	Amount of protein per dose	Safety		ED_{50}/ml in mice	Mean neutralizing indices in sheep sera following vaccination with inactivated RVF vaccine *								
			Baby mice	lamb		0	1 st Week	2 nd Week	4 th Week	Challenge	6 th Week	2 Months	4 Months	6 Months
Sterile	RVF antigen	5 ug/dose	Safe	Safe	0.014	0	1.4	1.9	3.1		2.4	2.8	2.4	2.0

ED_{50} / ml = effective dose fifty.
Protective level of NI is 2.00.

Table (3) : Protein Standard Curve

Optical Density (O.D.)	Protein Concentration (ug / 0.1 ml)
0.020	1 ug / 0.1 ml
0.040	2 ug / 0.1 ml
0.051	3 ug / 0.1 ml
0.082	4 ug / 0.1 ml
0.098	5 ug / 0.1 ml
0.120	6 ug / 0.1 ml
0.152	7 ug / 0.1 ml
0.162	8 ug / 0.1 ml
0.182	9 ug / 0.1 ml

Table (4) : Estimated of purified RVF antigen protein (ug / 0.1 ml) using Bradford method for standard curve of purified RVF antigen.

Purified RVF antigen dilution	Optical Density at A°595 by spectrophotometer	Protein content using the standard protein curve (ug / 0.1 ml)
1/200	0.124	6.2
1/400	0.064	3.1
1/8000	0.024	1.1
1/16000	0.010	0.5
1/32000	0.000	0.0

Table (5) : Protein content of reference purified RVF antigen and at O.D. measured by double sandwich ELISA.

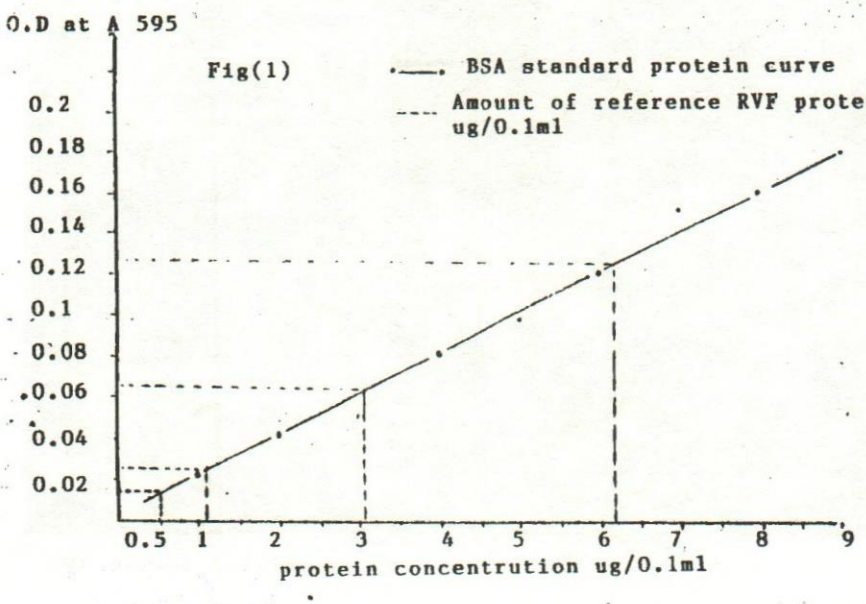
Protein content using the standard protein curve (ug / 0.1 ml)	ELISA O.D. at A°405
6.2	0.572
3.1	0.515
1.1	0.476
0.5	0.464
0.0	0.000

Table (6) : In-vitro ELISA for estimation of the amount of microgram of RVF viral protein per dose of vaccine and ELISA potency ratio.

Inactivated RVF vaccine	Batch No.	Average O.D. at 1/10 dilution of RVF vaccine	Amount of ug of RVF protein 0.1/ml	ELISA potency ratio of tested O.D. RVF # vaccine:reference one
*Reference RVF with alum gel.	137	0.462	0.4	Reference
Reference RVF without alum gel	137	0.464	0.5	Reference
Tested RVF vacc. with alum gel	1	0.470	0.8	0.01
	2	0.464	0.5	1.00
	3	0.462	0.4	1.00
	4	0.466	0.6	1.01
	5	0.464	0.5	1.00
	6	0.462	0.4	1.00
	7	0.462	0.4	1.00
	8	0.462	0.4	1.00
	9	0.466	0.6	1.01
Mean value		0.464	0.5	1.00
Control 1 cell protein		0.048	0.0	0.00
Control 2 alum gel		0.032	0.0	0.00

* A reference RVF vaccine validated by serological test and challenge of vaccinated sheep.

Protective potency ratio must be › 1.00.



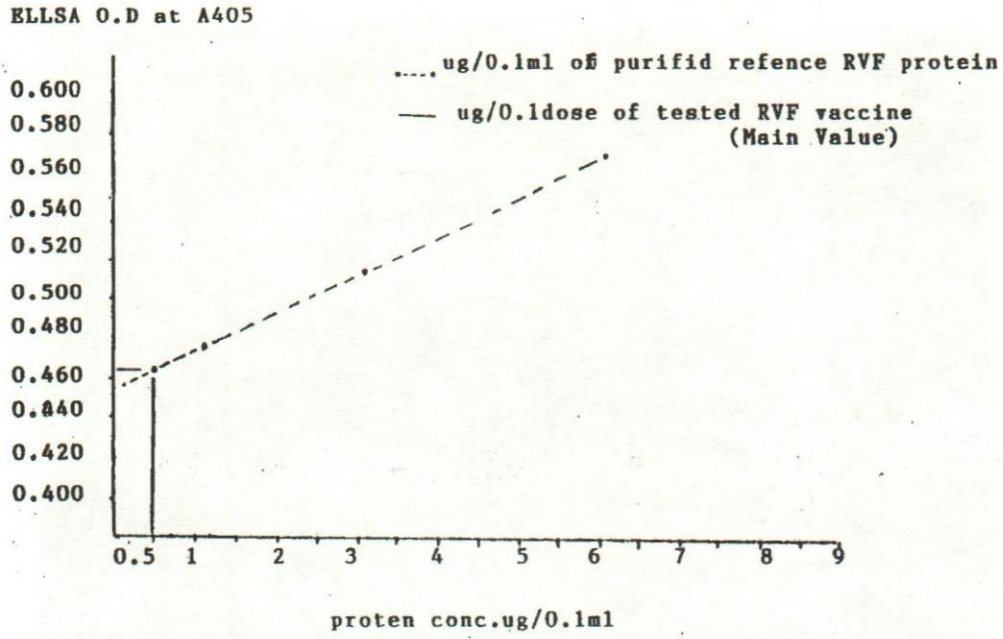


Fig (2)standard curve for reference RVF protein

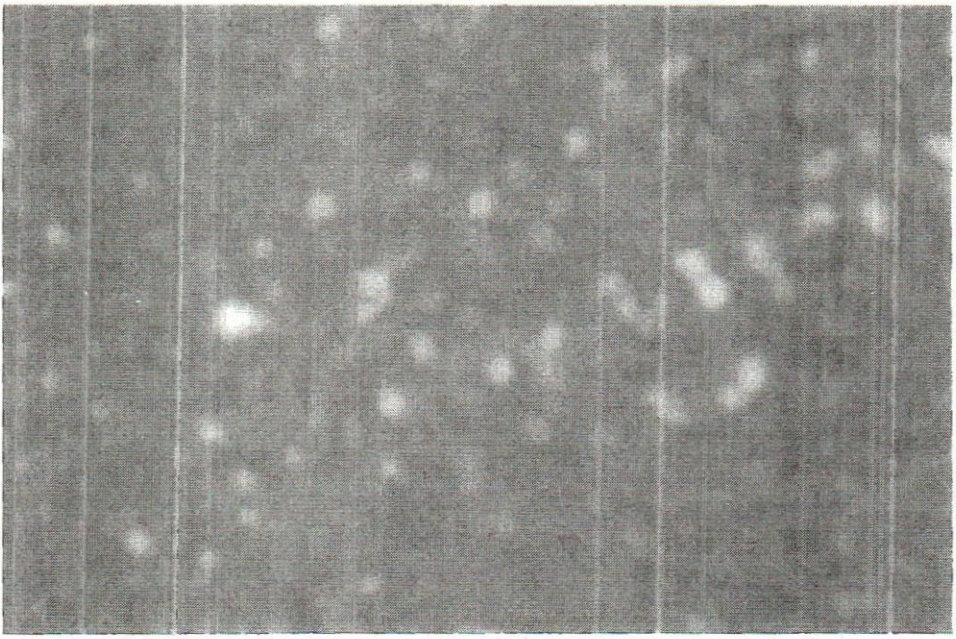


Photo (1) : BHK cells, 20 hours post infection with live attenuated RVF vaccines as done by IFA (X 160).

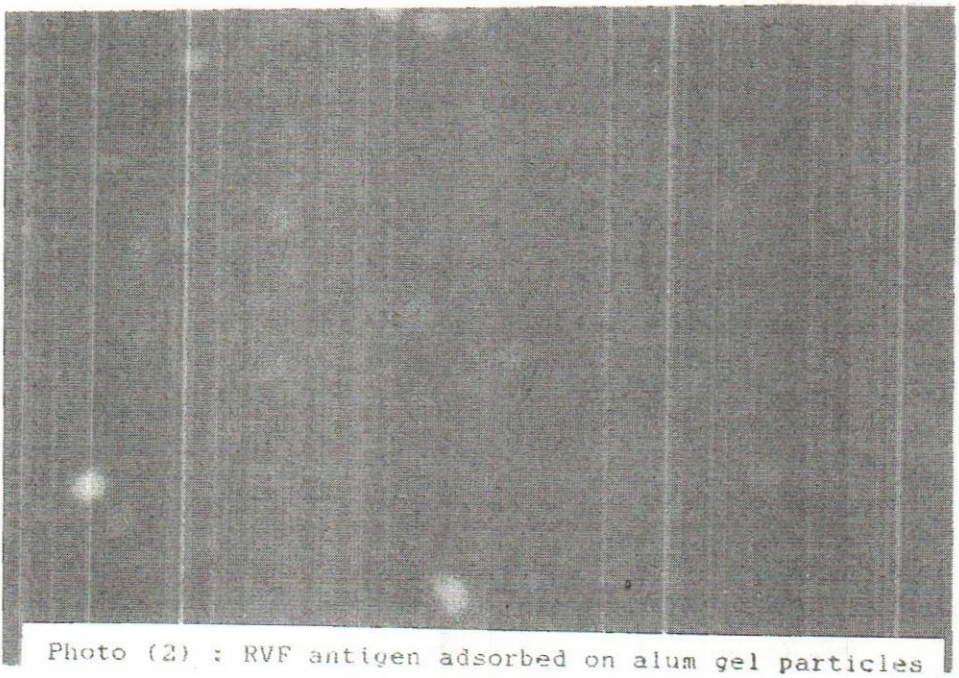


Photo (2) : RVF antigen adsorbed on alum gel particles of inactivated RVF vaccine as done by IFA (X 160).