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PREPARATION AND EVALUATION OF IMMUNOFLUORESCENT CONJUGATE AGAINST FMD VIRUS

M. M. ABD EL-ATY.; S. EL-KILANY.; M. SHAWKY.; and H. EL-WATANY

FMD Research Department.
Veterinary Serum and Vaccine Research Institute, Abbassia, Cairo.

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SUMMARY

A trial for the production and evaluation of an immunofluorescent conjugate against FMD virus infection in farm animals was conducted locally. Using serum of experimentally infected animals collected after 35 days post infection. This serum was treated with saturated ammonium suplhate for precipitation and isolation of g-globulins and estimation of total protein content. It was found that total protein content of serum used was 4 gm / 100 ml serum according to the protein content conjugation of the antibodies with fluorescein was applied in constant fluorescein / protein ratio. These labeled antibodies were purified and tested against free dye physicochemically for protein content, antibody specificity, labeling ratio and potency performance (titration). From the present study it was found that fluorochrome conjugate end point was 1/16 used in the test proper against positive samples for FMD virus. It is evident from the results obtained that all collected samples were giving positive results of high specificity against the prepared conjugate. The prepared fluorescent conjugated antiserum proved its efficiency for the localization of antigen of FMD virus in tissue sections and smears; also it is considered as rapid, easy and economic reagent for direct FA test used in viral diagnosis.

INTRODUCTION

Fluorescent antibody techniques have gained wide acceptance in virology, and have proved

especially useful in the study of animal viruses. Fluorescent antibody staining serves four main purposes, the cellular localization of viral antigens can be determined at the high microscope level (Watson and Coons, 1954; Spendlove et al., 1963). The temporal sequence of appearance after infection can be established for different viral antigens (Breitenfeld and Schafer, 1957). Virus infected cells which fail to produce viral progeny often still synthesize virus coded antigens which continue to harbor viral genetic material (Pope and Rowe, 1964; Tevethia et al., 1965). Coon's original technique (Coons and Kaplan, 1950; Coons 1958) has been modified numerous times and is now used as a routine tool in many laboratories (Nairn, 1962). An excellent comperhensive volume on fluorescent antibody methods has appeared recently and is highly recommended (Goldman, 1968).

Antibodies are clearly very useful for specifically recognizing antigens, but detecting, the antigen-antibody complexes can be difficult unless precipitation occurs. However "tagging" them with a label easily circumvents this problem. Such labels were originally radioisotopes such as ^{125}Iodine ^{125}(I). But fluorochromes are also used widely nowadays. Conjugation procedures are usually designed to produce an effective degree of antibody label conjugation whilst minimizing adverse effects on antigen recognition. The latter can result either from damage to the immunoglobulin molecule by the reagents used for recogntion or from introduction of bulky labels which sterically obstruct interaction with antigens (Alan Johnstone and Robinthrope, 1996).

Fluorescent dyes are commolny employed as labels in primary binding tests, the most important being fluorescein isothiocynate (FITC). FITC is a yellow component that can be bound to antibodies without affecting their reactivity. When irradiated with invisible ultraviolet light, FITC emits visible green light, so FITC-labeled antibodies are used in the direct and indirect fluorescent antibody tests (Tizard, 1996). Direct fluorescent artibody is used to identify the presence of antigen.

Antibody directed against a specific antigen such as bacterium or virus is first labeled with FITC, this test can identify viruses growing in tissue culture or in tissues from infected animals. It would be very expensive to purchase immunlogical reagents in the quantities required for carrying out virological techniques although a wide diversity of good quantity conjugates are commercially available, the techniques in the following studies will be invaluable if you have to prepare and standardize your own conjugate for specialist applications (Leslie Hudson and Frank C. Hay, 1989).

MATERIALS AND METHODS

Preparation of Y- globulin rich fraction of bovine antiserum against FMD virus (According to Leslie Hudson and Frank C. Hay, 1989).

Reagents and Equipment:

1- Ammonium sulphate $\{(NH_4)_2 SO_4\}$. ADWIC products of El-Nasr pharmaceutical chemicals Co. M. W. 132. 13.

2- Dilute ammonia solution or sulphuric acid.

3- Bovine serum of infected cattle with FMD virus collected after 35 days post infection.

4- UV spectrophotometer. Milton Roy Company, Spectronic 601, Made in USA. SALR 45227.

The following method was used:

1- 1000 gm ammonium sulphate were dissolved in 1000 ml distilled water at 50°C and allowed to stand overnight at room temperature. The pH was adjusted to 7.2 with dilute ammonia solution or sulphuric acid.

2- Serum was separated from the clotted whole bovine blood.

3- Serum was then diluted 1:2 with saline and saturated ammonium sulphate solution added to a final concentration of 45 % (v/v).

4- The diluted serum was stirred at room temperature for 30 minutes.

5- The precipitate was spun off (1000 gm for 15 minutes at 4°C).

6- The precipitate was then washed with 45% saturated ammonium sulphate and recentrifuged.

7- The precipitate was re-dissolved in the same volume of PBS as the original serum, centrifuged to remove any insoluble material and the γ-globulin re-precipitated using a final concentration of 40% saturated ammonium sulphate.

8- The precipitate was spun off and washed with 40% saturated ammonium sulphate.

9- After centrifuging, the precipitate was re-dissolved in a minimum volume of PBS.

10- The γ- globulin was dialyzed against 5 changes of PBS at 4°C and any precipitate centrifuged off.

11- The protein content of the γ-globulin was determined using the Biuret Method (1946).

Preparation of fluorochrome conjugated antisera of FMD virus (According to Leslie Hudson and Frank C.Hay, 1989).

Conjugation technique:

Materials and Equipment:

1- Bovine antiserum.

2- Saturated ammonium sulphate pH 7.2.

3- 0.25 M, pH 9.0 carbonate/bicarbonate buffer.

4- Sephadex G-25 column.

5- UV spectrophotometer.

6- Fluorescein isothiocynate (C_{21} H_{11} NO_5S) E, Merck, Darmstadt for microscopy (M.Gew. 389.39).

METHODS

1- The antiserum was precipitated of with 40% saturated ammonium sulphate as described above.

2- The γ-globulin fraction of antiserum was dialyzed against 0.25 M, pH 9.0 carbonate/bicarbonate buffer using a sephadex G-25 column.

3- The protein concentration of the solution was determined and adjusted to 20 mg/ml.

4- Fluorescein isothiocynate (0.05 mg per mg of total protein) was added and the solution mixed overnight at 4 °C.

5- The conjugated protein was separated from the free fluorochrome by passing the mixture down a G-25 sephadex column equilibrated with PBS or purified by dialysis against phosphate buffered saline in a cold room for 3 days. The outer fluid was changed at least twice daily.

Calculation of fluorochrome protein ratio:

This was done routinely every time a new conjugate was made.

The presence of the fluorochrome interferes with the OD of the protein at 280 nm. This is allowed for the formula:

$$\text{Fluoroscein: Protein ratio} = \frac{2.87 \times OD495\ nm}{OD280\ nm - 0.35 \times OD495\ nm}$$

If it was intended to use the conjugate to stain fixed material the F/P ratio should be low (2:1). However, antisera used to stain viable cells, where the specific and non-specific fluorescene is much weaker should have a higher conjugation ratio (2-4:1).

The prepared conjugate was stored frozen (-20°C or below) or filtered through millipore filter with average size of 0:45 μ before being kept in the refrigerator for several months.

Standardization of the prepared conjugate:

Two fold dilutions of the conjugation with PBS were made. Then from each dilution one drop of the conjugate was added to each fixed slide with known reference virus. Then was incubated at 37°C for 1 hour. Wash the slides with PBS and then add drop of glycerin buffer and examine under the microscope to detect the last diluton of conjugate which gave suitable reading could be used in the test proper as in Table (1) {i. e. dilution which gave a high specific fluorescence with low background}.

Direct staining technique of tested samples using the prepared conjugate according to (Peter K. Vogt, 1969):

Samples tested were:

1- Tissue culture infected with FMD virus

(BHK_{21} $CLON_{13}$ CELLS).

2- Baby mice samples (tissues).

3- Guinea pigs pad.

4- Oesopharyngal fluid from infected calves.

5- Epithelial tongue of infected calves.

6- Vesicular fluid from infected calves.

In this procedure the viral antibody was conjugated to FITC and served as a highly specific stain which combined with antigens in virus infected cells.

a- Fixation of infected cells:

The cover glass was rinsed in tris-buffered saline then drained and air-dried. Fixation was performed in acetone. for 5 minutes at room temperature. After drying, the cover glass was dipped into tris-buffered saline. Excess fluid was wiped off the back with absorbent paper and the cells became ready for staining.

b- Staining:

The cover glass was placed in humid chamber and the cell sheet was flooded with a drop of fluorescent antibody. Staining was accomplished in as little as 10 minutes but in some cases extensive staining period up to 24 hours has proved advantageous. Staining was carried out at room temperature or at 37°C. After staining the cells were washed twice, 5 minutes each time, with PBS and mounted in buffered glycerol. Control samples were included using non-infected cells (negative for FMD virus).

RESULTS

Table 1: Titration of conjugate.

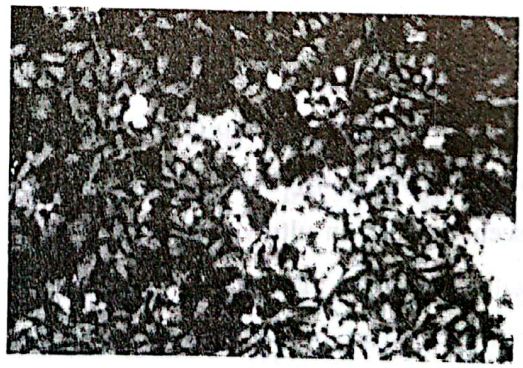
Samples	1/2	1/42	1/8	1/16	1/32	1/64	1/128	1/256
*Infected samles	+++	++	++	++	-	-	-	-
**Control samples	-	-	-	-	-	-	-	-

* Fixed T. C. slides infected with FMD virus type O1.
** Non infected T. C. smears using undiluted conjugate that gave negative results.
The endpoint of the prepared flurochrome conjugate is 1/16 used in the test proper (direct FA) as shown in Table (1).

Table 2: Testing of the prepared conjugate against infected samples with FMD virus.

Type of samples	Epith. Tongue	Foot pads (ulcer)	O.P.	T.C. infected cells	Guinea pigs (pads)	Baby mice tissues (infected)
Result of test used direct FA	++	++	++	++	++	++
* Control samples	--	--	--	--	--	--

* Non infected tissue culture using undiluted tested conjugate.
O.P. = Oesopharyngeal.
T.C. = Tissue culuture (BHK_{21} $clone_{21}$ cells).



Direct fluorescent staining technique for detecting FMD virus in T. C. (BHK21) cells using the prepared fluorochrome conjugate of dilution end point 1/16 (40 x objective).

V- Discussion and comments

The highest positive dilution of the prepared conjugate was 1/16. This dilution gave positive FA when used with infected different samples with FMD virus. For cytoplasmic staining or any fixed staining materials F/P ratio is lower due to low conjugation or protein concentration less than 0.5 mg/ml can be used (Talwar, 1983).

It is clear from our results in Tables (1 & 2) that direct FA test is highly sensitive and rapid test.

which detect even low numbers of antigen of FMD virus. This results in agreement with results obtained by (Tizard, 1996).

From the current literature, it seems that this is the first time to prepare fluorochrome conjugate for FMD virus diagnosis in Egypt.

So we recommend using fluorochrome conjugate for FMD virus samples collected from the field as rapid tool for diagnosis beside other serological tests.

Evaluation of the prepared conjugate was carrying out by placing a drop of the conjugate on filter paper strip and observes the fluorescence under an ultraviolet lamp. A good conjugate must show intensive color after 10 or 100 fold dilution as in this present study. The specificity of the staining has to be investigated in proper control experiments (Nowotny, 1979).

Evaluation of the prepared conjugate should be determined by 2 parts: 1st part physicochemical characterization for antibody content, antibody specificity, labeling ratio and presence of free dye. While 2nd part is evaluation of potency of conjugate by performance testing by making serial dilutions of conjugate to overcome the problems of nonspecific staining since most anti-immunoglobulin conjugates when tested by block titration against a known antibody containing serum give a plateau for the titer of the 1st serum over of dilutions of the conjugate. The extent of the plateau is a measure of the antibody potency of the conjugate and where there is adequate the conjugate dilution at limit of the plateau is well beyond the (lower) dilution of conjugate which may give rise to non specific staining (Johnson et al., 1978). Most of non-specific fluorescence appears to be caused by globulins which are too heavily conjugated with fluorescent isothiocynate (FITC) (Goldstein et al., 1961 and Herbert et al., 1967) so reducing the dye to protein ratio in the conjugation mixture alleviates this situation. The problem of nonspecific staining is also substantially reduced by using a pure y-globulin fraction for conjugation or by re-chromatography of the conjugate on DEAE-cellulose (Wood et al., 1965). In conclusion it may be stated that the prepared fluorochrome conjugated antiserum of FMD virus is rapid, easy, accurate and economic diagnostic reagent used in direct FA technique for identification and detection of FMD virus

samples collected from infected farm animals (i.e. may be used as rapid field diagnostic test for FMD virus detection without using indirect FA and other complicated serological tests.

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