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# Preparation and Evaluation of Anti-Rabbit IgY Peroxidase and FITC Conjugates

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In this investigation, a trial for preparation of anti-rabbit immunoglobulin-Y (IgY) peroxidase and fluorescein isothiocyanate (FITC) conjugates was conducted. Rabbit IgG was separated and injected into specific pathogen free (SPF) laying hens, from which their eggs were collected. The immunoglobulin-Y (IgY) was precipitated from the egg yolk of immunized hens by 33% saturated ammonium sulphate solution. The anti-rabbit immunoglobulin-Y (IgY) was purified by using affinity chromatography. The obtained globulins were divided into 2 parts, the 1st one was conjugated with peroxidase and the 2nd part was conjugated with fluorescein isothiocyanate. Both conjugates gave a strong positive result when diluted 1:2500 and 1:50, respectively. The results indicated that they could be used successfully for detection of antigens and antibodies.

## INTRODUCTION

Screening for persistent infection or antibody titres requires detection for both the presence of virus or viral antigen and determining antibody status. So, there is need for development of both inexpensive and rapid testing procedures.

ELISA test may offer the advantage of rapid turn around time and possibly lower costs for both detection of virus and antibody (Katz *et al.*, 1987). ELISA is serological marker technique. The principle of marker technique is the use of marked antibody to make the formation of specific immune complexes visible. The ELISA technique makes use of enzyme that has been coupled to antibodies, the so-called conjugates.

These conjugates are fixed to the specific immune complexes and the enzyme action on its substrate leads to an indicative colour reaction. Horseradish peroxidase is the most widely used enzyme label and has an affinity for cell membranes true.

Also, the FAT belongs to the marker techniques and was designed for the demonstration of antigens and antibody using fluorescein labeled antibody (conjugate). FA tests can be used for detection of antigens in various clinical specimens (Goldman, 1968).

Finally, the IgG horseradish peroxidase or FITC conjugates would cost a lot and take a lot of time and money to be exported from abroad. So, this work was aimed to prepare a locally anti-rabbit conjugates for ELISA and FA to be used for rapid detection of both antigen and antibody.

## MATERIAL AND METHODS

### Material

**1. Laboratory animals**

**a. Rabbit**

Ten apparently healthy New Zealand rabbits, 2.5-3 kg, were used in preparation of IgG from their serum.

**b. SPF laying hens**

Ten specific pathogen free (SPF) laying hens were used in preparation of IgY from their eggs.

**2. Chemicals used in precipitation of immunoglobulins**

a. Phosphate buffered saline (PBS):

It was used for sample dilutions and dialysis.
b. 33% Saturated ammonium sulphate $(NH_4)_2S0_4$.
c. 2 N NaOH for pH adjustment.

**3. Kit used for estimation of protein contents:**

It was obtained from Diamond Diagnostic Company, Egypt.

**4. Chemicals used in affinity chromatography:**

i. Sephadex G-25 column or dialysis tubing.
ii. Swelling buffer, 1 mM HCl buffer.
iii. Coupling buffer, $NaHCO_3$ buffer (0.1 M, pH 8.3) containing NaCl (0.5M).
iv. Blocking buffer, coupling buffer, containing 0.2 M glycine.
v. Washing buffer, 0.6M sodium acetate, 0.6M acetic acid, pH 4.0, containing 0.5 M NaCl.
vi. Starting buffer, 0.15 M phosphate-buffered saline (PBS), pH 7.2.
vii. Elution buffer, 0.1 M glycine-HCl buffer, pH 2.5
viii. Titration buffer, 4 M Tris-buffer.
ix. Bio-Rad test.

**5. Chemicals used in peroxidase conjugation:**

a. Horseradish peroxidase (HRPO):

It was supplied by Sigma Chemical Co., St. Louis, USA, Cat. # P-6782.
b. 0.2 M and 0.01 M sodium carbonate.
c. Sodium borohydride 4mg/ml.
d. 0.001 M sodium acetate.
e. Sodium periodate, 0.1 M.
f. PBS.

**6. Chemicals used in FITC conjugation:**

a. Fluorescein iso-thiocyanate: It was obtained from Merck Co., Denmark, Cat. # 24546.
b. 0.5 M carbonate / bicarbonate buffer.
c. Phosphate buffered saline (PBS).
d. Sephadex G-25 column or dialysis tubing.

**Methods:**

**I. Preparation of rabbit IgG**

The test was performed as the method described by Peter (1969) and Henry (1974).

Separation of gamma globulin from serum was conducted by repeating precipitation with ammonium sulfate, at a final concentration of one-third saturation. Usually three precipitations suffice to separate the gamma globulin in fairly pure form. The salts were removed by using dialysis bag.

**II. Immunization schedules of laying hens:**

Immunization of laying hens was done as the method described by Hudson and Hay (1991).

- A laying hen was immunised with 100 µg foreign protein antigen (rabbit IgG) dissolved in 0.5ml sterile saline added to 0.5 ml Freund's complete adjuvant. The mixture was injected subcutaneously at both sides of the chest.
- The inoculation was repeated after 2-3 weeks with the same amount.
- It was necessary to re-immunise a third time 2-3 weeks after the second boosting.
- Eggs were collected 2 weeks after the last injection.

**III. Preparation of IgY from eggs of immunized hens:**

Anti-rabbit antibodies were extracted from the egg yolk preferably by ammonium sulphate precipitation

according to Peter (1969) and Henry (1974).

The protein concentration of IgY suspension was measured with the Biuret method (Henry, 1974) and stored at -20°C.

## IV. Affinity Chromatography

The Affinity Chromatography was carried out as the method described by Hudson and Hay (1991), using G-25 Sepharose column:

*1. Swelling and washing the gel:*

The required amount of freeze-dried gel powder was swollen for 15 min. in 1 mM HCL. One gram freeze-dried powder gave a gel volume of approximately 3.5 ml. The gel was then washed with coupling buffer (5 ml per gram dry gel) and immediately transferred to a solution of the ligand, which had been dissolved and dialysed in coupling buffer before. A ratio of gel to buffer 1:2 gave a suitable suspension for coupling.

*2. Coupling the ligand:*

The mixture containing ligand and swollen gel was rotated end-to-end for 2 hr at room temperature or overnight at 4oC.

*3.Blocking excess active groups:*

The gel was transferred to buffer with blocking agent as 0.2 M glycine pH 8.0, then incubated at 4oC for 16 hours.

*4. Washing the product:*

The adsorbent was washed alternately with high and low pH buffer solutions four or five times as coupling buffer followed by acetate buffer then coupling buffer.

*5. Storage of coupled protein:*

Protein coupled to CN8r-activated Sepharose 4B was stored at 4°C in the presence of a bacteriostatic agent such as thiomersal or sodium azide (0.1 M).

*6. Chromatography stage:*

The immunoadsorbent was poured into the column and equilibrate with PBS. The sample was run to be purified, dissolved in PBS, through the column. The unbound protein was washed from the column until the absorbance measured in a flow through UV cell is <0.1, otherwise wash with 200 ml PBS.

*7. Elution:*

0.1 M glycine-HCL buffer (pH adjusted to 2.3 -2.5) was added to the top of the column and collect the effluent when protein is first detected. When protein was no longer detectable, collecting the effluent was stopped.

*8. Titration of sample:*

pH of the elution buffer was adjusted to 7.2 using 4 M Tris-buffer. The eluted sample was examined for presence of protein using Bio-Rad test.

*9. Preparation and storage of samples:*

The positive eluted protein samples were concentrated in a dialysis tubing with either sucrose, polyethylene or carboxymethyl cellulose. The precipitate was spin off and determined the protein content of each sample. Samples were stored in aliquots at -20°C

## V. Preparation of FITC conjugated antisera: (Klugerman, (1965)

1. The concentrated gamma-globulin (IgY) protein fraction of the rabbit antiserum was determined and adjusted to 10-30 mg/ml with normal saline.
2. Antiserum solution was kept on ice bath and carbonate/bicarbonate

buffer was added to the extent of 10% by volume.
3. 0.05 mg fluorescein isothiocyanate was added per mg of total protein.
4. Then it was stirred gently overnight at 4oC.
5. The conjugated protein was separated from the free fluorochrome by gel filtration using G-25 sephadex column.

**VI. Conjugation of horseradish peroxidase to immunoglobulin**

It was conducted using the periodate method according to Wilson and Nakane (1978). Anti-rabbit-immunoglobulin serum (IgY) was dialyzed against sodium carbonate buffer (0.01 M) overnight at 4oC and the concentration was adjusted to 8mg/ml.

**VII. Indirect enzyme linked immunosorbent assay (Indirect ELISA)**

The indirect ELISA was carried out to evaluate the prepared conjugate using IDEXX ELISA kit against Infectious Bronchitis (IB) virus.

**VIII. Fluorescent antibody technique (FAT)**

The FAT was carried out to evaluate the prepared conjugate according to Habel and Salznan (1969).

## RESULTS AND DISCUSSION

This investigation was conducted to prepare local specific anti-rabbit sera conjugated with horseradish peroxidase and fluorescein isothiocyanate to be used in ELISA and FAT. Where there is an increasing demand to such product for rapid and accurate diagnosis of some diseases and estimated of antibody titres after vaccination with different vaccines.

The prepared peroxidase conjugate was examined by ELISA method to determine the optimal dilution of locally prepared peroxidase labeled antibodies as shown in table (1). The results revealed that the highest dilution of the prepared conjugate was 1:2500 when applied on rabbit serum samples previously proved to be positive for infectious bronchitis virus. Also, this conjugate was used in parallel with a standard imported one which gave positive result until dilution 1:10000.

Indirect FAT was used to demonstrate the presence of specific anti-rabbit antibodies in the prepared FITC conjugate. The test was applied on serum samples containing anti-IB antibodies prepared in rabbit. FAT revealed good positive result up to a dilution 1:50 (Table 2 and Fig. 1) and weak positive at dilution 1:80 comparing with the imported one which gave good positive result at dilution 1:80 and weak positive at dilution 1:120.

So, it is possible to use this prepared conjugate in FAT either to detect viral antigens or antibodies in rabbit sera to obtain a rapid and accurate diagnosis. This result came in agreement with that of Brian and Hillor (1996).

In conclusion, the prepared anti-rabbit conjugates with peroxidase and fluorescein isothiocyanate were of good quality and could be used successfully in the detection of viral antigens or antibodies present in rabbit serum.

**Table (1): S/P ratio of the prepared anti-rabbit serum conjugated with horseradish peroxidase**

| Conjugate Dilutions | Imported conjugate | Local prepared conjugate |
|---|---|---|
| 1:5 | 18.6 * | 2.9 |
| 1:50 | 16.4 | 2.6 |
| 1:100 | 14.4 | 1.8 |
| 1:200 | 13.74 | 1.6 |
| 1:250 | 11.4 | 1.6 |
| 1:500 | 8.2 | 0.8 |
| 1:1000 | 6.9 | 0.3 |
| 1:2000 | 5.2 | 0.3 |
| 1:2500 | 2.9 | 0.3 |
| 1:5000 | 2.6 | 0.1 |
| 1:10000 | 1.6 | 0.03 |

* S/P ratio = $\frac{\text{Sample - Negative}}{\text{Positive – Negative}}$

N.B. S/P ratio greater than 0.2 is considered positive.

N.B. Control rabbit negative serum gave 0.01 and 0.05 with local prepared and imported conjugates respectively when used at dilution 1:5.

**Table (2): Titration of the prepared anti-rabbit serum conjugated with fluorescein isothiocyanate**

| Dilutions | Imported conjugate | Local prepared conjugate | Control Serum |
|---|---|---|---|
| 1:5 | ++++ | ++++ | - |
| 1:10 | ++++ | ++++ | - |
| 1:20 | ++++ | ++++ | - |
| 1:30 | ++++ | ++++ | - |
| 1:40 | ++++ | +++ | - |
| 1:50 | +++ | +++ | - |
| 1:80 | +++ | ± | - |
| 1:100 | ++ | - | - |
| 1:120 | ± | - | - |

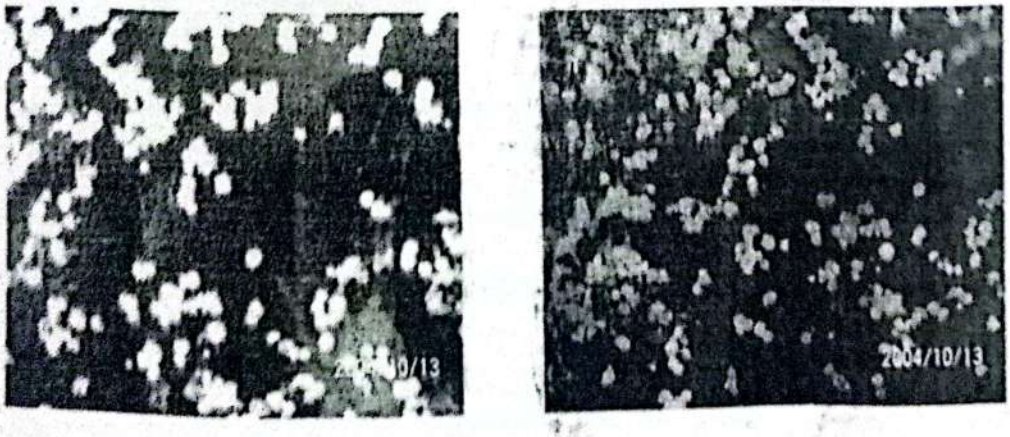


Fig. (1): Positive green FA reaction

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