

**PREPARATION OF ANTI-CAMEL IMMUNOGLOBULIN-G CONJUGATED
WITH FLUORESCIN ISOTHIOCYANATE AND ALKALINE
PHOSPHATASE**

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ABSTRACT

Two anti-camel immunoglobulin-g (IgG) conjugates were prepared, anti-camel IgG conjugated with fluorescein isothiocyanate (FITC) and anti-camel IgG conjugated with alkaline phosphatase.

Camel IgG was prepared by precipitation of camel sera with 50 % saturated ammonium sulphate and after that IgG was separated by ion exchange chromatography. Anti-camel IgG was prepared by immunization of goats with camel IgG then anti-camel IgG was precipitated by saturated ammonium sulphate, and anti-camel IgG was separated by ion-exchange chromatography.

Anti-camel IgG was conjugated with fluorescein isothiocyanate (FITC) and with alkaline phosphatase. Sensitivity and specificity of prepared conjugate were evaluated. The conjugates were used for diagnosis of tuberculosis among camels by enzyme-linked-immunosorbent assay (ELISA) and indirect fluorescent antibody techniques (IFA).

INTRODUCTION

For diagnosis of infectious diseases, serological techniques are simple-inexpensive and as rapid diagnostic tests for many of these infectious diseases. Enzyme linked immunosorbent assay (ELISA) and fluorescent antibody techniques are the most sensitive and specific serological tests if compared with other tests. Both tests need labeled anti-species IgG with enzymes as horseradish peroxidase or alkaline phosphatase or other enzymes for ELISA or with fluorescein isothiocyanate (FITC) for different fluorescent antibody techniques. Labeled anti-species IgG is most available in market for almost type of animal species except camels, where the market is deficient in labeled anti-camel IgG either with enzymes or fluorescein isothiocyanate.

The present investigation was directed for preparation of anti-camel IgG labeled with alkaline phosphatase enzyme and with fluorescein isothiocyanate. The following stages of work were planned to fulfill the target plans:

1. Separation of suitable amounts of camel IgG through precipitation of immunoglobulins from camel sera by ammonium sulphate and purification of the class IgG from other classes by ion exchange chromatography.
2. Preparation of anti-camel IgG antibodies by injection of goats by the prepared and purified camel IgG..
3. Purification of anti-camel IgG from sera by ion-exchange chromatography which is an economic and suitable purification technique in course of commercial production of labeled anti-camel IgG.
4. Labeling of anti-camel IgG with calf intestinal alkaline phosphatase enzyme.
5. Labeling of anti-camel IgG with fluorescein isothiocyanate.
6. Evaluation of the validity of the prepared labeled anti-camel IgG wither for purity, sensitivity, specificity and storage.
7. Application of the prepared labeled anti-camel IgG for diagnosis of some tuberculosis in camels under field conditions.

MATERIAL AND METHODS

Blood was collected by jugular puncture immediately before slaughtering from normal health camels to be ensure free from any obvious disease. Serum was separated from clotted blood camel according to Goers (4). Camel immunoglobulins (Igs) were precipitated with 50 % saturated ammonium sulphate (SAS) according to Ungur-Waron et. al. (13). Ammonium sulphate was removed by dialysis against

phosphate buffer saline (1 x PBS pH 7.2) for several days at about 4°C, the dialyzate was changed every 12 hours and the dialysis was continued till no ammonium sulphate was detected. Igs solution was concentrated approximately ten times by polyethylene glycol (PEG) according to Ingham, (5). The protein content of Igs were determined by Lowery's et. al. (7).

The IgG was separated by ion-exchange chromatography on Di-ethyle-amino-ethyle cellulose according to Fahey and Terry (3). IgG was detected by agar gel precipitation test (AGPT) according to Ouchterlony, (9), the anti-camel sera which used for detection of IgG

by AGPT was kindly provided by Prof. Dr. Rafky EL-Karamany, Egyptian Organization of Sera and Vaccines, Agouza.

Anti-camel IgG were prepared by goat immunization, ten mg of prepared camel IgG was thoroughly mixed with equal volume of freund's complete adjuvant (FCA) was injected subcut in goat, four booster doses mixed with equal volume of freund's in complete adjuvant (FICA) subcut in the goat, each booster dose was injected one week interval. The first sample was taken before the immunization began to prepare a suitable control for further tests, 1st booster, 2nd booster, then weekly. The blood sample was taken to separate the serum to detect the protein content on spectrophotometer and AGPT.

Goat anti-camel IgG was conjugated with alkaline phosphatase according to O'Sullivan and Marks (8) as following, 3mg of alkaline phosphatase were added to 1.5 mg of goat anti-camel IgG in PBS, gluteraldehyde was added to 0.20 % with gentle vortex, the mixture were incubated for 2 hours at room temperature, desalted by chromatography on sephadex G-25 and finally stored at 4°C after sterile filtration or addition of sodium azide.

Also anti-camel IgG was conjugated with fluorescein isothiocyanate (FITC) as following, FITC stock solution were prepared at 1 mg/ml in DMSO, 0.1 ml of FITC solution was added dropwise to 10 mg of anti-camel IgG in 1 ml carbonate-bicarbonate buffer (pH 9.5), mixing and were left 2 hours in the dark at room temperature, Unreacted FITC was removed by sephadex G-50 medium, eluting with 1 X PBS, the first coloured peak was collected (this contained the labeled IgG) and was concentrated to the original volume that was applied. The conjugate was stored in the dark at 4°C.

Evaluation of prepared anti-camel IgG conjugated with alkaline phosphatase and FITC were subjected to ELISA and IFA, respectively. By using thirty blood sample, 19 of them were collected from tuberculous camel (examined by tuberculin

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test, PM lesions, and bacteriologically proved to be tuberculous) and 11 samples were collected from camels proved to be free from tuberculous as negative control.

RESULTS

The yield of whole Igs after 50 % SAS precipitation was 2000 ml then after concentration by using PEG was 800 ml. It was found that the protein content of Igs was 1.7 mg/ml. By ion exchange chromatography, two peaked chromatograms. The fraction for each peak from several runs was pooled, dialyzed and concentrated. The yield of obtained camel IgG was about 59 ml. The protein content was 5.5 mg/ml while the total amount of IgG was 324 mg.

Blood sample were taken from each goat at the zero day, at the 1st booster, at the 2nd booster dose and weekly until the time of bleeding. Total globulins of goats was increased about 8.6 times from zero day until the slaughtering time. The anti-camel sera were tested by AGPT serum were collected from all goats and purified as described previously. The protein content of anti-camel IgG was 3.2 mg/ml.

Results of evaluation of freshly prepared goat anti-camel IgG alkaline phosphatase by ELISA cleared that, the anti-camel IgG can be used till 1:50 dilution in PBS for capture the antibody in positive camel sera. The application of anti-camel IgG as conjugate at dilution 1:100 or more gave false negative result. The result of stability anti-camel IgG alkaline phosphatase by ELISA cleared that the conjugate is still active and able to capture the antibody in the positive camel sera when stored at 4°C or 20°C till 1 year. The conjugate can be applied at dilution 1:50 and 1:25 when stored at 6 months at 4°C and 20°C, respectively. The conjugate after 9 months storage (4°C or 20°C) can be applied as 1:25, dilution when stored at 4°C for 1 year and as 1:2 dilution when stored at -20°C. Application of anti-camel IgG alkaline phosphatase conjugate in comparison with protein-A- conjugate for examination of camel sera, the result cleared that, the prepared conjugate gave 100 % sensitivity while the application of protein-A-alkaline phosphatase gave 84.2 % sensitivity.

Evaluation of anti-camel IgG conjugated with FITC gave good positive (+++) till dilution 1/16. The reaction gradually decreased at dilution 1/32 (++) and gave negative result with positive sera at dilution 1/64. The application of prepared conjugate gave sensitivity 100 % at dilution 1/16.

DISCUSSION

Several methods including gel filtration, ion-exchange chromatography and affinity chromatography are available for the purification of immunoglobulins. The

procedure followed in the present study was the initial precipitation of camel globulins by 50 % SAS for precipitation of Igs. Many authors used 50 % SAS in

precipitation of camel Igs, Ungar et. al. (13), Reddy and Gridhar (12), Ramesh and Reddy (10).

The precipitated camel Igs were extensively dialyzed against 1 X PBS for several days to remove ammonium sulphate and the end of dialysis was detected by absence of white precipitate which appear in dialyzate containing ammonium sulphate tested with few drops of 10 % barium chloride (6). Concentration of Igs was performed according to Ingham.

In the present study, camel IgG was separated according to Fahy and Terry (3), by anion-exchange chromatography. Ion-exchange chromatography with cellulose derivatives is recognized as one of the most efficient and useful methods for the separation and purification of proteins.

It can be concluded that purification of anti-species IgG by affinity chromatography is more efficient than with DEAE-cellulose column chromatography but it is more expensive. This result was in agreement with Chan (2) who recorded that the affinity chromatography is simple, one step, highly efficient method for IgG purification. Purified camel IgG with DEAE-cellulose chromatography was investigated by AGPT. These two lines may indicate the separation of IgG into two subclasses IgG1 and IgG2 (1). After purification of camel IgG, goats were inoculated to produce anti-camel IgG. Goats are widely used for production of specific antibodies (11), in the present study. It can be observed that the increase in total globulins observed that the increase in total globulins reached 9 times the amount of globulins at zero day in goats.

Detection of anti-camel IgG in sera of immunized animals was performed by AGPT. Goat anti-camel Igs were subjected to ion-exchange chromatography on DEAE-cellulose for purification of anti-camel IgG.

As the labeled anti-camel Igs with enzymes, for ELISA are not available in commercial level, the present investigation was directed for producing labeling anti-camel IgG with alkaline phosphatase enzyme. In the present work, anti-camel IgG were labeled by alkaline phosphatase enzyme bind by gluteraldehyde using affinity chromatography technique by sephadex-G-25 anti-camel IgG labeled with 3 mg alkaline phosphatase gave a yield of 3 ml purified labeled anti-camel IgG.

Indirect method of ELISA was done to evaluate and measure the optimum dilution of the prepared conjugate, the result cleared that the highest dilution of alkaline phosphatase labeled anti-camel IgG that gave OD 3 fold the mean of negative sera was at 1/50. This dilution which can be used for using the prepared conjugate in ELISA. Also stability of the prepared conjugate was detected by investigation of preserved conjugate at -20C. and 4 C by testing of sera collected from tuberculous camels. Also the result revealed that prepared conjugate have sensitivity of 100 %, all positive and negative camel sera were tested with protein A conjugate. The result showed that protein-A conjugate failed to detect 3 cases of the positive sera. In the present study this means that sensitivity of protein-A conjugate was less 14.8 % than anti-species.

Another method of labeling anti-species is through FITC. Purified anti-camel IgG was labeled by FITC. FITC isomer is the best dye available for the immunofluorescence in terms of fluorescence efficient, stability and combining capacity with protein. In the present study, the pH available

during FITC binding with anti-camel IgG was 9.5 reached by carbonate-bicarbonate buffer. After protein labeling free dye was removed by sephadex gel filtration using sephadex G- 50 medium column.

The validity of the locally prepared anti-camel IgG conjugated with FITC was evaluated by testing 30 sera collected from healthy and diseased camels with tuberculosis. The result cleared that when the sera examined by indirect immunofluorescent using the anti-camel IgG were positive by giving a fluorescent reaction reaching 3 plus at dilution 1/16 of conjugate. These results revealed the high sensitivity of the in direct immunofluorescence in diagnosis of tuberculosis in camels. Specificity of the test may not be satisfactory as many related bacteria like atypical mycobacteria, and corynebacteria may sensitized. The animals giving non-specific results.

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تحضير ضد الامينوجلوبولين - ج للجمل مرتبط بالفلورسين ايزوثيوسيانيت
والفوسفاتيز القاعدى

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القاهرة

تم تحضير اثنين من ضد الامينوجلوبولين للجمل (ضد الامينوجلوبولين للجمل مرتبط بالفلورسين ايزوثيوسيانيت و ضد الامينوجلوبولين للجمل مرتبط بالفوسفاتيز القاعدى .
تم تحضير الامينوجلوبولين - ج للجمل بواسطة ترسيب سيرم الجمال بـ 50% سلفات الامونيوم المشبع وبعد ذلك تم فصل الامينوجلوبولين - ج بواسطة تبادل الايون الكروماتوجرافى . ثم تحضير ضد الامينوجلوبولين - ج بواسطة تحصين الماعز بـ الامينوجلوبولين - ج للجمل ثم بعد ذلك تم ترسيب ضد الامينوجلوبولين - ج للجمل بواسطة سلفات الامونيوم المشبع , وتم فصل ضد الامينوجلوبولين - ج للجمل بواسطة تبادل الايون الكروماتوجرامى . ثم ربط ضد الامينوجلوبولين - ج للجمل بـ الايزوسيانيت الفلورسنتى وبالفوسفاتيز القاعدى ثم تقييم حساسية وخصوصية المرتبط المحضر .
المرتبط المحضر استخدم فى تشخيص السل بين الجمال بواسطة الاليزا وبالطريقة الغير مباشرة لحد الجسم الفلورسنتى .