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## **PREPARATION OF C-REACTIVE PROTEIN LATEX REAGENT OF HUMAN SERUM**

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### **ABSTRACT**

C-reactive protein (CRP) is an acute phase reactant the concentration of which increases significantly following tissue injury or inflammation. It is present in the albumin fraction of the serum of patients and is antigenic upon injection into rabbits. We report here the development of a local latex reagent coated with rabbit anti-CRP. For this purpose CRP was isolated from pooled sera of patients and injected into rabbits. CRP rabbit antiserum was purified by using ammonium sulfate precipitation. Followed by passing on ion exchange chromatography (Diethylaminoethyl "DEAE" cellulose). Then, latex particles were coated with purified antisera. The prepared CRP latex reagent agglutination was observed upon the addition of patients' sera and compared with that of normal sera. In addition, it was evaluated against a standard CRP latex reagent. The prepared reagent gave positive results (100%) with patients' sera and the positive control of the standard kit. While, the negative control of the standard kit and 30% of the normal sera gave negative results.

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keywords: CRP, latex agglutination.

## تحضير محلول لاتكس لبروتين C المنفعل من مصّل الإنسان

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### الخلاصة

يعد البروتين المنفعل C احد بروتينات الطور الحاد الذي يزداد تركيزه بشكل ملحوظ بعد اصابة النسيج والالتهاب. يوجد هذا البروتين في جزء الالبومين من مصّل المرضى وذو فعالية مستضدية عند حقنة في الارنب. تم في هذا البحث تطوير محلول لاتكس محلي مغلف بالاضداد النوعية لبروتين C المنفعل المحضر في الارنب ولهذا الغرض تم عزل البروتين المنفعل C من خليط امصال مرضى و تم حقنة في الارنب, و من ثم تمت تنقية الاضداد النوعية للبروتين المنفعل C المحضرة في الارنب باستخدام الترسيب بكبريتات الامونيوم ثم بالمبادل اللايوني DEAE, بعد ذلك تم تغليف محلول الاتكس بالاضداد النوعية النقية. لوحظ حدوث التلازن مع محلول الاتكس المحضر بعد اضافة مصّل المرضى له بالمقارنة مع المصل الطبيعي. قبيست بعد ذلك العدة المحضرة مع محلول لاتكس قياسي, اذ اعطى المحلول المحضر محليا تفاعلا ايجابيا مع امصال المرضى والسيطرة الايجابية للعدة القياسية بنسبة 100% بينما اعطت تفاعلا سلبيا مع امصال المرضى بنسبة 30 % وكذلك مع السيطرة السالبة للعدة القياسية.

## **INTRODUCTION**

Since the discovery of CRP by Tillet and Francis in 1930 (1), this acute phase serum protein has been used as a marker of infection, inflammation and tissue necroses. It has been established that CRP is synthesized by liver cells (2).

Several *in vitro* studies have indicated that CRP may play an important role in modifying inflammatory and immune responses (3). CRP in complex with an appropriate ligand can activate the classical complement pathway (4). It has also been shown to enhance phagocytosis (5), bind to certain subpopulations of T-cells interfering with some of their functions (6), and inhibit platelet aggregation and mediator release (7).

Studies have emphasized the clinical value of CRP quantitation in a wide variety of diseases including systemic lupus erythematosus (8), rheumatoid arthritis (9), myocardial infarction (10), and pneumonitis (11). The CRP level, when compared to other acute phase parameters, is useful in the management of infection, especially recommended in neonatal sepsis and meningitis (12, 13). It is an effective diagnostic and disease monitoring tool, thus rapid and reliable quantitative assays for CRP are essential. Furthermore, reliable estimates of moderate levels of CRP are needed to help differentiate between viral and bacterial infections as well as for the evaluation of therapeutic effectiveness (12).

Because of their rapidly and ease of performance, semiquantitative CRP latex slide agglutination tests are increasing in availability and popularity. The present paper describes the preparation of CRP latex reagent.

## **MATERIALS & METHODS**

### **Isolation of CRP**

CRP was isolated from pooled sera (15 ml) having a CRP titer of 1:4, obtained from patients with acute infections as described by Macleod and Avery (14)

with some modifications. These sera were brought to 33% saturation with ammonium sulfate, centrifuged and the supernatant raised to 50% saturation with the same salt, centrifuged and the supernatant was brought to 75% then 100% saturation, respectively. The precipitate (after 75% saturation), recovered by centrifugation was dialyzed against three liters of 0.01% CaCl<sub>2</sub> in physiological saline. Protein concentration was determined by spectrophotometer (Schimadzu-Japan) at 280 nm; while the presence of CRP was determined by adding a constant amount of a standard CRP latex reagent to serial dilutions of the isolated protein. The preparation of protein was stored at 4°C and used as immunizing antigen.

#### **Production of anti-CRP antibodies**

Rabbits were immunized by seven intravenous injections on each of seven consecutive days with 1ml of the prepared antigen containing 1mg/ml of protein. After a week two booster doses were given, the interval between injections was one week. Sera were collected seven days after the last injection and preserved by freezing at -20 °C (14, 15). Skin test was performed in order to check the sensitization of the rabbits to the injected antigen, by injecting 0.1ml (1mg/ml) of the isolated protein subcutaneously. The second day a positive reaction was determined by the appearance of erythema and edema at the site of injection (1).

#### **Purification of CRP antibodies**

This was achieved by precipitation with ammonium sulfate (33%). Then samples were collected and dialyzed against phosphate buffer pH 8.0. The protein concentration was measured by spectrophotometer. The sample which was precipitated by ammonium sulfate was applied on DEAE cellulose column. Fractions were measured by spectrophotometer. The fractions that showed the highest absorbance (peak) were collected and dialyzed against 1500 ml of distilled water at

4°C. The volume was concentrated and the concentration of protein was determined by spectrophotometer (17).

#### **Coating latex particles with purified CRP antibodies**

Latex particles (0.8  $\mu$ , Difco-USA) were sensitized by adding equal volumes of CRP antibodies (concentration 3 mg/ml) to a 10% v/v suspension of latex particles and rotating for one hour. The suspension was left at 4°C overnight, before centrifugation at 13000 rpm. Then the supernatant was discarded and the pellet resuspended to 5% concentration in phosphate buffer, pH 8.0 and mixed by vortex (18). Then 0.1% of bovine serum albumin was added to block any remaining latex protein binding sites (17). Later on, sensitized latex solution was stored at 4°C.

Latex agglutination test was performed by adding 20  $\mu$ l of serum with a drop of the prepared CRP latex reagent on a microscopical slide with rotation for up to two minutes. Positive result was determined by forming visible agglutination.

#### **Evaluation of latex reagent**

The prepared CRP latex reagent was evaluated by using 12 serum samples of patients in comparison with standard CRP kit (Omega Diagnostics Limited- UK). In addition 10 normal serum samples and positive and negative controls of the standard kit were also tested with the prepared latex reagent.

### **RESULTS & DISCUSSION**

The most rapid simple method available for CRP determination is the slide agglutination test. The CRP used for the immunization of rabbits was prepared from blood obtained from cases with acute infections. It was found that CRP is combined with lipid in the blood and lipid-protein complex precipitate in the presence of calcium ion if the salt concentration is low (19). Thus the precipitate that formed after saturation with ammonium sulfate can be dialyzed with a diluted solution of

calcium chloride without loss of the reactive protein. This method was suitable for obtaining the C-protein relatively free from the normal serum proteins and in a form suitable for testing its antigenicity in rabbits.

Saturation with ammonium sulfate was applied for the isolation of CRP (Table 1). This resulted in a purification factor of 6.3 and a recovery of 66.7 %. The CRP content so prepared was 0.048 mg/ml.

The results of the CRP content demonstrated that the reactive protein isolated by ammonium sulfate is antigenic and in this form is capable of inciting in rabbits the production of antibodies specifically reactive with the homologous protein in the native state in which it exists in the serum of infected patients.

The CRP antiserum was firstly purified by ammonium sulfate. The concentration of protein was 32.2 mg/ml. Ammonium sulfate precipitation is the most widely used technique for the preparation of a crude immunoglobulin fraction from whole serum. It is often useful as a first step in isolation as many unwanted serum proteins, for example albumin, will remain in solution when immunoglobulins are precipitated. Ammonium sulfate is important for concentrating immunoglobulins from dilute solution (17).

The precipitated sample was applied on DEAE-cellulose column. The results showed one peak (Figure 1). The concentration of CRP-antibodies purified by DEAE-cellulose was 0.73 mg/ml. Ion exchange chromatography is one of the most popular methods for the separation of serum proteins and the isolation of immunoglobulins. For preparing IgG alone, the DEAE-cellulose is equilibrated under conditions of pH and ionic strength which allow all the serum proteins to bind except IgG (17).

The results showed that the presence of CRP could be detected in patients' sera when the latter was diluted 1 part in 4, when the prepared CRP latex reagent was

used. The prepared reagent gave positive results (100%) with patients' sera and the positive control of the standard kit. Although, the results of the prepared latex gave concordance results as with the standard kit, false positive agglutination was also detected with normal sera (30%), when were used.

The ambiguous agglutination for false positive finding with the prepared CRP latex reagent might be due to heterogeneity of CRP. It was described that anti-human CRP produced in rabbits could contain two subpopulations of specific antibodies (20). Thus, differing proportions of unlike antibody subpopulations coating the latex particles might well give varying agglutination patterns. It is recommended to produce the anti-CRP antibodies from other animal species, such as sheep or goat.

The observed differences in results between the standard and prepared CRP latex reagents might depend on the amount and properties, such as avidity or specificity, of the coating antibodies. The agglutination and / or prozoning produce by one sort of antibody may be masked by another type (21).

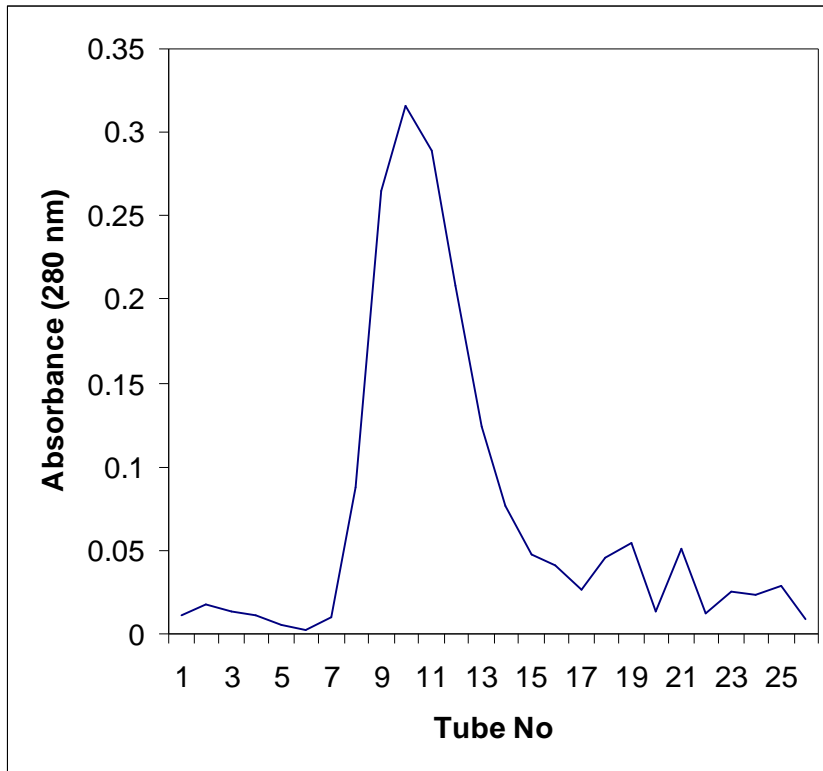
Furthermore, other explanation of the false positive results, is that rheumatoid factor (RF) can interfere with the detection of CRP by latex agglutination, causing false positive results, especially when RF is present in high titers. This was expected since RF reacts with the IgG anti-CRP used to sensitize the latex particles. In addition, another observation, using radioimmunoassay for IgM RF, indicate that rabbit IgG serves more sensitively as the antigen than either horse, sheep or goat IgG (22).

**Table 1: Measurement of C-reactive protein (CRP) from human serum**

Saturation with Ammonium Sulfate	Volume ml	Protein conc. mg/ml	CRP content* (activity mg/ml)	Total activity mg	Specific activity	Recovery %	Purification factor
Crude	15	86.2	0.024	0.36	0.00027	100	1
33 %	5	39.4	0.006	0.03	0.00015	8.33	5.6
50 %	5	20	0.012	0.06	0.0006	16.66	2.2
75 %	5	28.2	0.048	0.24	0.0017	66.66	6.3
100 %	5	0.521	0	0	0	0	0

\*CRP was determined by CRP agglutination kit.





**Figure 1: Elution profile of C-reactive protein antibodies from DEAE- cellulose column of bed volume 25 x 2.5 cm, flow rate 25 ml/hour, column 25 x 2.5 cm, 3ml/tube and the antibodies were eluted with 0.5M phosphate buffer pH 8.**

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