Henry Ford Hospital Medical Journal

Volume 18 | Number 3 Article 2

9-1970

Quantitative Determination of C-Reactive Protein (CRP) by Micro-Double Diffusion Technic

Hajime Hayashi

Gerald A. LoGrippo

Mary Perry

Follow this and additional works at: https://scholarlycommons.henryford.com/hfhmedjournal

 Part of the Life Sciences Commons, Medical Specialties Commons, and the Public Health Commons

Recommended Citation

Hayashi, Hajime; LoGrippo, Gerald A.; and Perry, Mary (1970) "Quantitative Determination of C-Reactive Protein (CRP) by Micro-Double Diffusion Technic," *Henry Ford Hospital Medical Journal* : Vol. 18 : No. 3 , 155-164.
Available at: https://scholarlycommons.henryford.com/hfhmedjournal/vol18/iss3/2

This Article is brought to you for free and open access by Henry Ford Health System Scholarly Commons. It has been accepted for inclusion in Henry Ford Hospital Medical Journal by an authorized editor of Henry Ford Health System Scholarly Commons.

Henry Ford Hosp. Med. Journal
Vol. 18, No. 3, 1970

Quantitative Determination of C-Reactive Protein (CRP) by Micro-Double Diffusion Technic

Hajime Hayashi, Ph.D., Gerald A. LoGrippo, M.D. and Mary Perry*

C-reactive protein (CRP) determinations in serum have been evaluated by two methods: the capillary tube method (presently in use) and the double diffusion technic (a newly developed procedure standardized in this laboratory). Advantages of the double diffusion technic are (1) quantitation of CRP in milligrams per 100 ml of serum, (2) differentiation of CRP from non-specific precipitates often found in patients' sera, and (3) increased sensitivity. A normal range of CRP levels has been established by analyzing serum from 750 supposedly healthy blood bank donors. Ninety-five percent of the donor population possess less than 1.0 mg of CRP per 100 ml of serum (53% are negative, 23% with trace quantities and 19% with 0.2-1.0 mg per 100 ml of serum). CRP serum levels above 1.0 mg per 100 ml are significant values for clinical interpretation.

Although the nature and significance of C-reactive protein (CRP) in serum remains an enigma, numerous investigators have described the presence of CRP in the serum of patients with a wide variety of diseases, especially some with acute and chronic inflammatory conditions.[1-8] The diseases include particularly rheumatic fever, rheumatoid arthritis, myocardial infarction, liver diseases, and various diseases of the digestive system.

Methods for the detection and determination of CRP include: detection of capsular swelling of the pneumococcus;[9] visual estimation of density and width of precipitation bands;[2] quantitative spectrophotometric measurements;[10] and quantitative gel-diffusion analysis, expressed either as serum titer or as positive and negative findings.[8,11,12] The most widely used clinical procedure is the capillary tube precipitation method of Anderson and McCarty.[2]

This report describes a micro-double diffusion method for the quantitation of CRP in milligrams (mg) per 100 milliliter (ml) of serum. The advantage of this technic permits the differentiation between CRP and other non-specific precipitable reactants often found in sera of patients with inflammatory processes.

Materials and Methods

Serum specimens were obtained from blood bank donors, laboratory personnel and diseased patients with a variety of clinical entities. All CRP determinations by the two methods studied were made on fresh sterile sera.

*Division of Immunology and Virology, Department of Pathology.

Anti-CRP rabbit serum and CRP standard were obtained from a commercial source (Difco Laboratories, Detroit, Mich.). These materials were standardized by the micro-double diffusion technic described later.

Capillary precipitation method for CRP. The capillary precipitation method of Anderson and McCarty[2] was employed for the comparative study with the micro-double diffusion technic. Total capillary precipitation is measured in millimeter (mm) and reported clinically in values from 1-plus (1 mm) to 6-plus (6 mm). The amount of precipitation is read after two hours of incubation at 37° centigrade (C) and re-read after 18 hours at the refrigeration temperature (4-6° C).

Micro-double diffusion technic for CRP quantitation. Materials and equipment used in this procedure are described elsewhere.[13] The micro-double diffusion in agar technique utilizes a five-well plastic template* arranged with a central well and four peripheral wells, which are equal distance from the central well (4 mm). Each well has a capacity of 0.025 ml. Molten agar (1% in phosphate-saline buffer) is placed on a glass microscope slide, allowed to gel slightly, and overlayed with the plastic template. Anti-CRP serum is placed in the central well and two-fold dilutions of the patient's serum are placed in the peripheral wells (Fig 1). After 24-36 hours incubation in a moist chamber, the templates are removed. The distance measurements are made from the center of the central well to the inner edge of the precipitation bands through a projecting viewer at the 24 times magnification (Fig 1). The distance of the band from the central well is inversely proportional to the CRP concentration in the diluted serum in the peripheral wells.[13,14] This principle is applied for the CRP quantitation.

*Available from M. H. Specialties Co., 20254 Woodward Avenue, Mt. Clemens, Michigan.

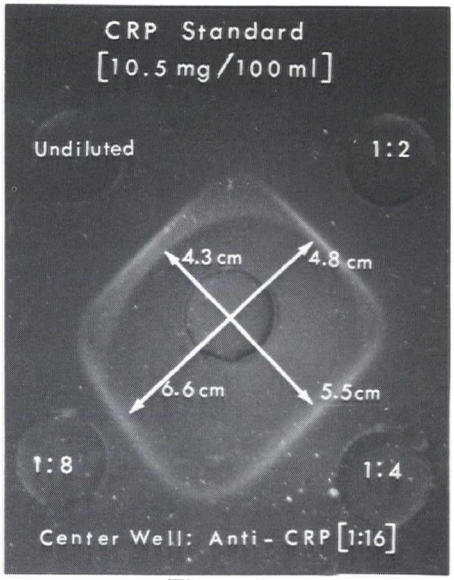

Figure 1

Quantitation of CRP (expressed in mg per 100 ml of serum) is extrapolated from the distance measurements indicated by the arrows (see Fig 2). The eccentric square results from anti-CRP in the center well and the different concentrations of CRP in the peripheral wells. The distances are inversely proportional to the CRP concentrations in the wells when precipitated at the zone of equivalence.

Results

Both methods being compared are immunochemical procedures. Before establishing the quantitative procedure, it became necessary to demonstrate the purity of the commercial anti-CRP rabbit serum (antibody) and CRP standard (antigen).

Quantitative Determination of C-Reactive Protein (CRP)

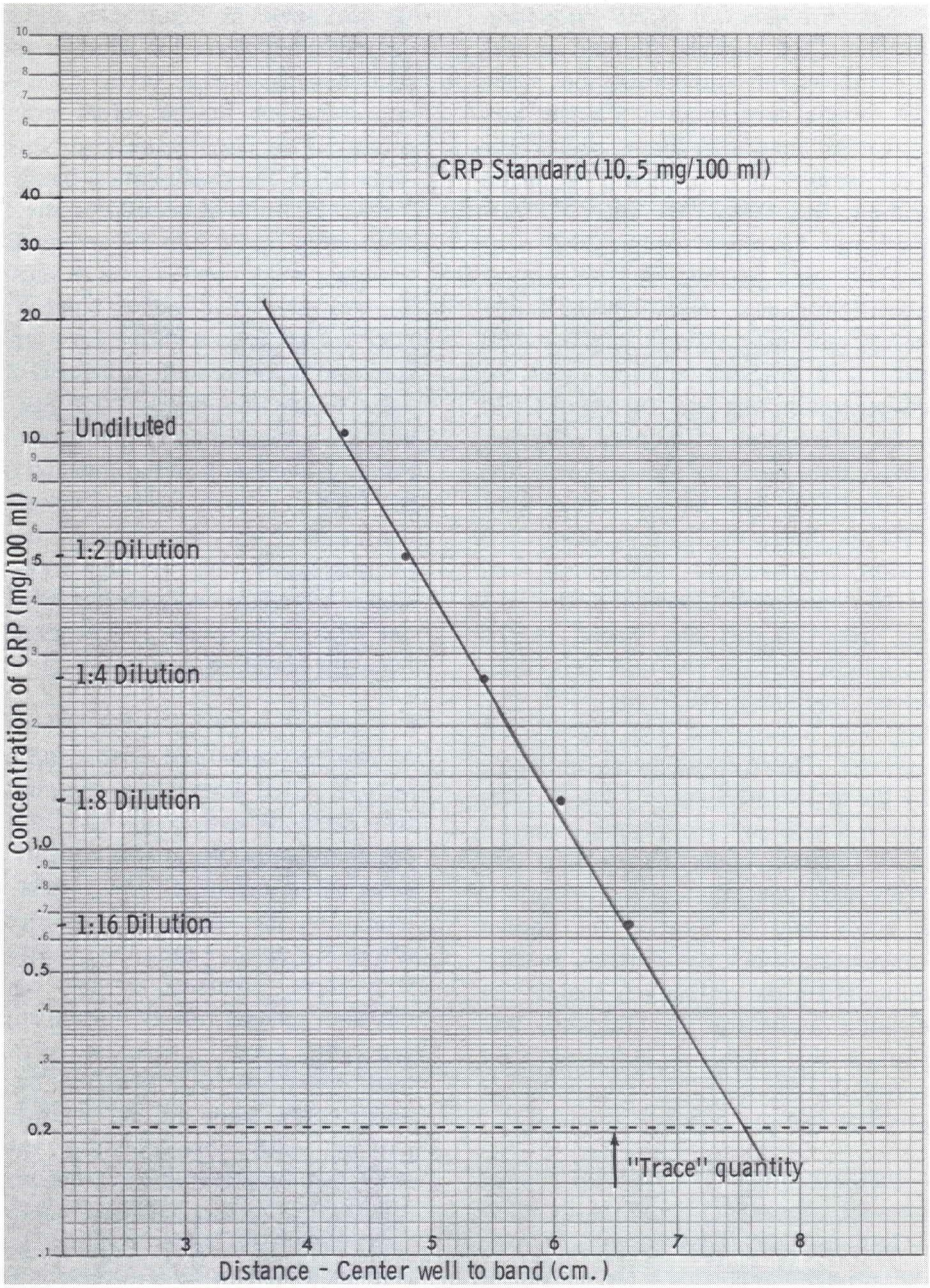

Figure 2

The curve established with purified CRP (10.5 mg per 100 ml plotted on the ordinance) and distance readings obtained from Figure 1. Standard curve used to extrapolate distance measurements in unknown serum sample. Quantities below 0.2 mg per 100 ml are not reliable measurements and are expressed as "trace" quantities.

Establishment of CRP standard. Purified CRP material (human source) is suitable for standardization when the protein content gives a single precipitation band with the specific anti-CRP serum in the double diffusion technic. In addition, the anti-CRP serum must be mono-specific and must not show any precipitable band against normal human serum. With these materials, one may assume that the total protein in this standard represents the concentration (mg per 100 ml) of CRP.

In establishing a standard reference curve, data must be obtained with the purified CRP and mono-immune anti-CRP as shown in Figure 1. Anti-CRP serum is placed in the central well (1:16 dilution) and purified CRP (10.5 mg per 100 ml) are placed in the peripheral wells in two-fold serial dilutions: undiluted, 10.5 mg; 1:2, 5.2 mg; 1:4, 2.6 mg and 1:8, 1.3 mg per 100 ml. The measurements indicated by the arrows (4.3 cm, 4.8 cm, 5.5 cm and 6.6 cm) are the results obtained from different concentration of CRP. With these data, the curve in Figure 2 is established by plotting the known concentration of total proteins in each dilution (on the ordinance) against the distance found (on the abscissa). The straight line obtained indicates good correlation between the concentration of CRP and the distance obtained.

Determination of CRP in clinical serum specimens. Quantitation of CRP in patients' sera must be measured at the zone of equivalence as shown in Figure 3-b. In Figures 3-a, 3-b and 3-c, the varied precipitation patterns shown are the results obtained when the anti-CRP concentration is kept constant while the CRP in the patient's serum varies. In Figure 3-a, the precipitation bands are too wide for quantitative measurements because the CRP concentrations in the serum are unusually high. In Figure 3-c, on the other hand, the precipitation bands are too weak or absent because of low concentrations of CRP in the serum. Even though a weak band is seen, it must be demonstrated that such a band is the CRP by its fusion with the known CRP standard incorporated in the test. Such weak bands (less than 0.2 mg per 100 ml) are reported as *trace* quantities. Figure 3-b shows how the concentration of CRP in each serum sample is converted from the average of six measurements (three readings from two slides) to milligram quantities of CRP per 100 ml of serum by extrapolation from the standard curve (Fig 2).

Comparison of CRP determinations by capillary tube and double diffusion technic. CRP levels in serum by the two methods are given in Table I. Serum samples from 140 patients are grouped according to the results obtained by the capillary tube method (presently used) and are compared with those obtained by the double diffusion technic (newly developed). A generally good correlation between these two tests is emphasized by the underscored slope in the number of patients with corresponding values. However, results from the two procedures show three interesting comparisons. The *first* is in the negative group with the capillary tube method. Of the 40 patients found negative for CRP by the capillary tube method, 17 (43%) of these were negative and 23 (57%) possess trace or low measurable quantities (0.2 to 2.0 mg per 100 ml)

TABLE I

COMPARISON OF C-REACTIVE PROTEIN DETERMINATIONS BY CAPILLARY TUBE AND DOUBLE DIFFUSION TECHNICS

Capillary Tube Method	Total Tested	Number of samples in respective groups given by double diffusion method					
		Negative	Trace*	0.2-2.0	2.1-5.0	5.1-10	> 10 mg%
Negative	40	17	12	11	0	0	0
±	19	7	8	4	0	0	0
1+	20	1	3	8	8	0	0
2+	13	1	1	1	6	4	0
3+	9	0	0	2	5	1	1
4+	11	0	0	1	1	3	6
5+	14	0	0	0	0	2	12
6+	14	0	0	0	0	0	14

* Trace; less than 0.2 mg per 100 ml of serum (lowest limit of reliable measurement).

by the double diffusion technic. This demonstrates the increased sensitivity of the quantitation of CRP. The *second* comparison is seen in the groups indicated by 2-plus or less in the capillary tube method. These include nine patients that are negative by the double diffusion technic. The measurable precipitates in the capillary tube method are not specific for CRP (as is demonstrated in Fig 4). Although the visible precipitation band labelled BBD-372 (Fig 4) is also precipitable in the agar, it can be seen that this precipitate is not identical with the know CRP standard band. Note the difference (Fig 3) between the continuous fusion in the bands between CRP standard

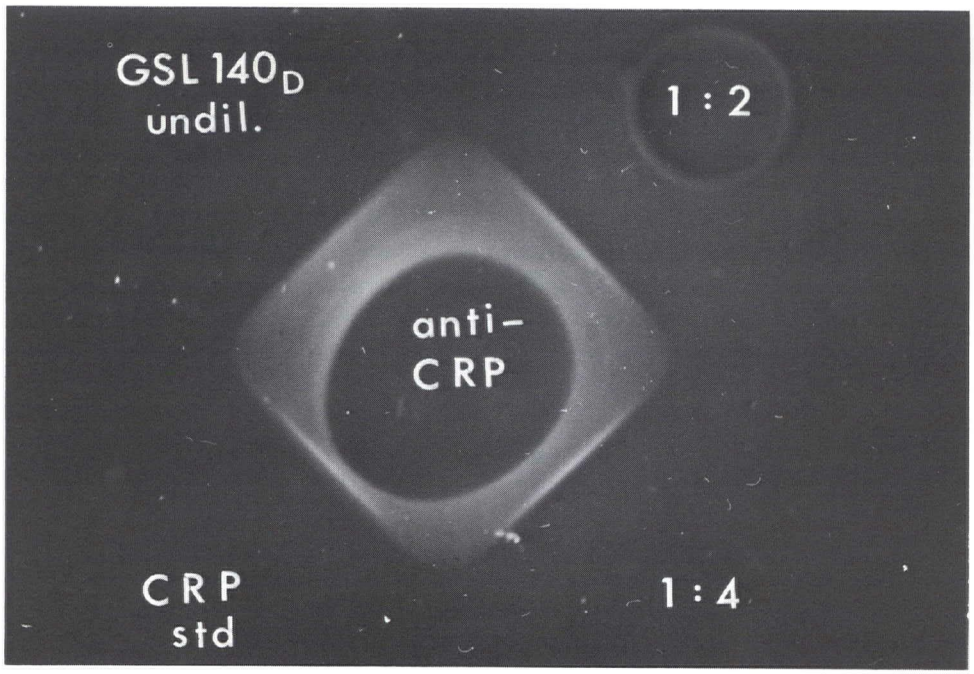

Figure 3-a

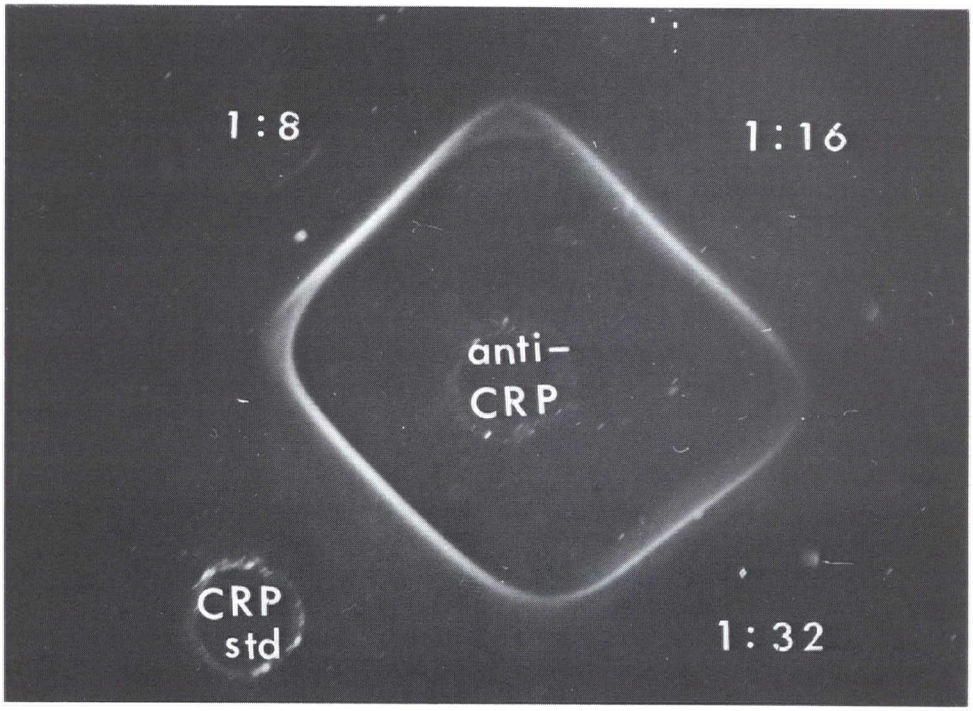

Figure 3-b

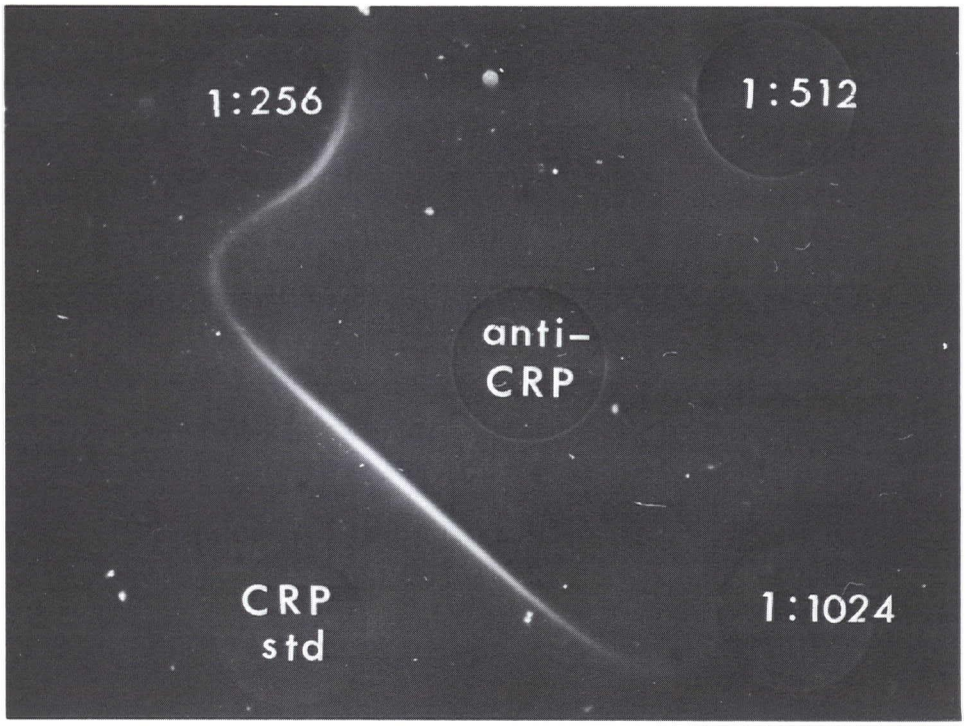

Figure 3-c

Precipitation pattern seen in clinical specimens when CRP is present in abnormally large quantities (Figure 3-a); in quantities suitable for quantitation (3-b) at the zone of equivalence; and minute quantities not suitable for quantitation (3-c) but indicative of trace quantity and proof of the CRP band of identity.

and unknown serum dilution of 1:256. This denotes bands of identity between the known and unknown precipitates. Because this differentiation can not be made by the capillary tube method, false reports are given. The *third* point of interest in the two methods is shown in the 6-plus group of the capillary tube method. Six-plus or greater measurements do not indicate the CRP concentration in serum. Whereas in the double diffusion technic, the 14 patients varied in CRP concentrations from 10 mg per 100 ml to 96 mg per 100 ml of serum. CRP levels in serum offer a more precise clinical index to the inflammatory processes and physiological stresses in the patients, particularly when studied in serial intervals with serum samples.

CRP determinations in supposedly healthy population. In order to establish a relative normal range for clinical interpretation, 750 blood bank donors were studied to determine CRP levels in serum by the micro-double diffusion technic. Table II shows that 76%* of the population demonstrated negative to trace quantities of CRP, 19% possessed 0.2 to 1.0 mg per 100 ml of

*Percentages expressed in nearest whole number.

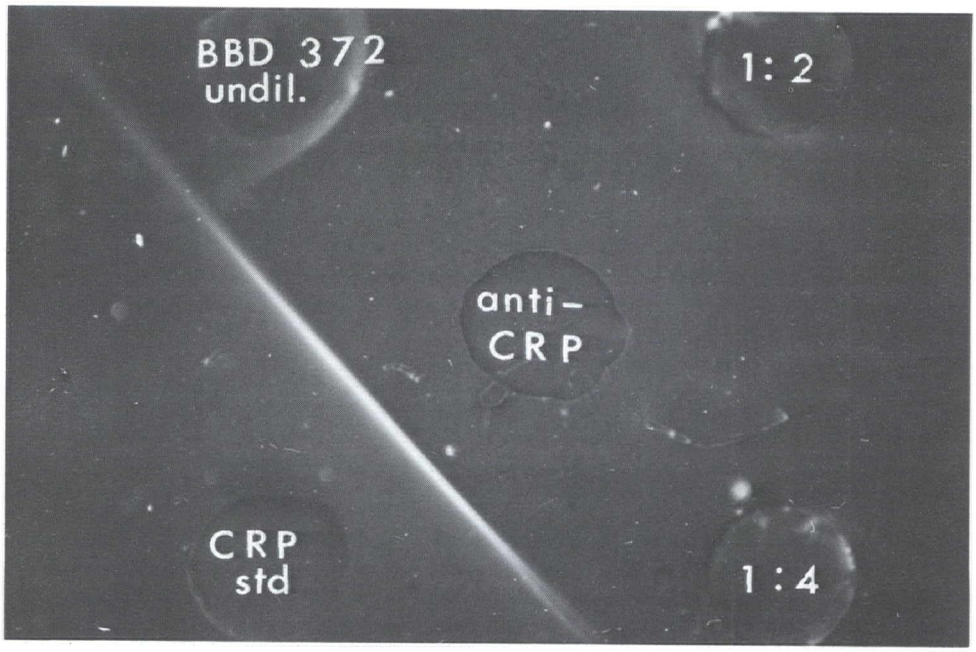

Figure 4

Precipitable material in patient's serum which gives false results in the capillary tube method. These can be identified in the double diffusion method as shown in this figure. The precipitate (BBD372) does not fuse with a known CRP standard.

TABLE II

SERUM C-REACTIVE PROTEIN LEVELS IN 750 BLOOD BANK DONORS

(Double-Diffusion Technic)

mg per 100 ml	Negative	Trace*	0.2-0.5	0.6-1.0	1.1-3.0	>3.1
Number of Donors	396	172	82	59	32	9
	(52.8%)	(22.9%)	(10.9%)	(7.8%)	(4.3%)	(1.2%)
	75.7%		18.7%		5.5%	

* Trace; less than 0.2 mg per 100 ml of serum (lowest limit of reliable measurement).

serum, and 6% possessed 1.1 mg per 100 ml of serum or greater. The 95 percentile (76% and 19%) includes serum CRP levels of 1.0 mg per 100 ml or less in the population. This range could be considered an acceptable range for a normal clinical standard. Values above 1.0 mg per 100 ml should be considered significant for clinical interpretation.

Discussion

Although the appearance of C-reactive protein (CRP) in serum is indicative of a non-specific response to inflammatory processes and various physiologic stresses, quantitative determinations offer an excellent means for evaluating hidden infectious processes. The advantage of quantitating abnormal quantities of CRP in patient serum, particularly when present in excess quantities, is often helpful in patient care and management.

The double diffusion technic for quantitating CRP levels in serum offers several advantages to the clinicians:

(1) Quantitation on a milligram basis for indications of increase or decrease quantities of CRP in inflammatory or stress processes, especially rheumatic fever and kidney diseases.[15, 16]

(2) Differentiation of non-specific precipitable material in serum which is not identifiable by the capillary tube method.

(3) Increased sensitivity of the quantitation for detecting minute quantities of CRP in serum and for quantitation of excessive amounts of CRP beyond the 6-plus values expressed in the capillary tube method.

REFERENCES

1. Tillett, W. S., and Francis, Jr., T.: Serological reactions in pneumonia with a non-protein somatic fraction of pneumococcus. *J Exp Med* 52:561-71, Oct 1930.
2. Anderson, H. C., and McCarty, M.: Determination of C-reactive protein in the blood as a measure of the activity of the disease process in acute rheumatic fever. *Amer J Med* 8:445-55, Apr 1950.
3. Rozansky, R., and Bercovici, B.: C-reactive protein during pregnancy and in cord blood. *Proc Soc Exp Biol Med* 92:4-6, May 1956.
4. Philipson, L., and Tveteras, E.: C-reactive protein in infancy. Its appearance during the first year of life, transplacental passage, and electrophoretic pattern. *Acta Paediat Scand* 46:1-9, Jan 1957.
5. Hornung, M., and Morris, T. M.: Electrophoretic determination of C-reactive protein which appears following surgery. *Proc Soc Exp Biol Med* 111:25-30, Oct 1962.
6. Nilsson, L. A., and Hanson, L. A.: Studies on the presence of C-reactive protein during the pre- and neonatal period. *Acta Pathol Microbiol Scand Suppl* 154:255-7, 1962.
7. Anzai, T., et al: Immunoelectrophoretic patterns of C-reactive protein in serum and in ascitic fluid from patients with cancer. *Proc Soc Exp Biol Med* 120:94-8, Oct 1965.
8. Mishimura, E. T.; Takahara, S., and Hokama, Y.: Interrelationship of catalase and C-reactive protein in acatalasemic erythrocytes. *Arch Biochem* 126:121-30, Jul 1968.
9. Lofstrom, G.: Nonspecific capsular swelling in pneumococci. A serologic and clinical study. *Acta Med Scand Suppl* 141:1-98, 1943.

10. Wood, H. F., and McCarty, M.: Measurement of C-reactive protein in human sera. Comparison of the clinical tests on the basis of a quantitative method. *J Clin Invest* 30:616-22, Jun 1951.

11. Libretti, A.; Kaplan, M. A., and Goldin, M.: Precipitin analysis of C-reactive protein by gel diffusion. *Proc Soc Exp Biol Med* 90:481-4, Nov 1955.

12. Fukuda, M.; Heiskell, C. L., and Carpenter, C. M.: A method for quantitative determination of C-reactive protein using gel-diffusion. *Amer J Clin Path* 32:507-12, Dec 1959.

13. Sharpless, N. S., and LoGrippo, G. A.: A standardized immunochemical method for quantitative determination of the immunoglobulins in serum. *Henry Ford Hosp Med Bull* 13:55-77, Mar 1965.

14. LoGrippo, G. A.; Manson, G., and Sharpless, N.: Immunoglobulin levels in serum of normal infants and pre-school children as determined by immunochemical analysis. *Henry Ford Hosp Med J* 15:247-58, Fall 1967.

15. Hayashi, H., and LoGrippo, G. A.: Australia antigen, immunoglobulins, C-reactive protein in virus hepatitis. *Bacteriol Proc* 70:117-8, 1970.

16. LoGrippo, G. A.; Anselm, K., and Hayashi, H.: Serum immunoglobulins and five serum proteins in extrahepatic obstructive jaundice and alcoholic cirrhosis. (Presented in part to the 4th International congress of gastroenterology, Copenhagen, July 12, 1970. Submitted for publication.)