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LATEX AGGLUTINATION TEST : A SIMPLE, RAPID AND PRACTICAL METHOD FOR BOVINE SERUM CRP DETERMINATION

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A semi-quantitative latex agglutination test for bovine serum CRP levels has been established by mixing diluted serum (or diluted standard serum) with a 1% latex suspension containing 0.489 μm latex particles coated with affinity-purified antibody at a ratio of 20 $\mu\text{g}/\text{mg}$ latex. The agglutination was performed on a glass slide in a moist chamber at room temperature with 45 min. incubation. This test is reliable, reproducible and the results correlate with those of the single radial immunodiffusion (SRID) test. The effect of low temperature storage on CRP concentration revealed a 30% degradation of CRP during 2 years storage at 4°C. The possible role of EDTA addition to prevent a decrease in serum CRP concentration by freezing and thawing is also discussed.

Keywords : latex agglutination, C-reactive protein, bovine, acute phase protein

INTRODUCTION

C-reactive protein (CRP) is known as a classical acute-phase protein in the human, the serum level of which increases several hundred fold during inflammation and / or tissue injuries¹²⁾. CRP has been observed in various species of animals ranging from the invertebrate horseshoe crab (*Limulus polyphemus*) to mammals including humans and cattle, with common properties of molecular structure and ligand binding specificity^{1,2,7,8,13,14,16)}. This indicates that CRP has been highly conserved over an extremely long evolutionary period. Although its actual function *in vivo* is still unclear, it has been suggested that one of the functions of CRP is to bind specifically to foreign pathogens and damaged cells of the host, thereby enhancing their elimination by interacting with humoral and / or cellular immune effectors in the

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blood^{4,18)}.

Bovine CRP was first reported in 1987 by Maudsley et al.⁸⁾ and its physicochemical properties and changes in serum levels were elucidated by Morimatsu et al.^{9,10)}. Recently, a new method for bovine CRP purification has been established¹⁷⁾. To date, only the single radial immunodiffusion (SRID) test or enzyme-linked immunosorbent assay (ELISA) have been used to study bovine serum CRP levels^{9,10,17)}. This paper describes the latex agglutination test as a rapid, simple and practical method for the clinical determination of bovine serum CRP levels.

MATERIALS AND METHODS

Latex particles : Polystyrene latex particles (JAPAN SYNTHETIC RUBBER Co., Ltd., Immutex S080, Tokyo, Japan) with a diameter of $0.489 \mu\text{m}$ were suspended at a concentration of 2% in 0.01 M glycine buffer (pH 7.3) containing 0.75% NaCl and kept at 4°C until use.

Preparation of anti-bovine CRP antibody (Ab) : The globulin fraction of rabbit anti-bovine CRP serum (30 ml) previously prepared¹⁰⁾ was precipitated at a concentration of 33% saturated ammonium sulfate. The pellet was then dissolved and dialyzed against 0.05 M Tris-phosphate buffer (pH 8.0) and the dialyzed sample was applied to a DE-52 (WHATMAN Inc., Clifton, NJ, USA.) column (2.5×20 cm) equilibrated with starting buffer at a flow rate of 20 ml/hr. Solid ammonium sulfate was added to the fractions at 33% saturation at 4°C and left overnight. The pellet was dissolved in 5 ml Dulbecco's phosphate-buffered saline (PBS) at a final protein concentration of 15.6 mg/ml, determined by absorption at 280 nm. with $1_{\text{cm}}^{1\%} = 14.3$, and kept at -80°C until used. In addition, anti-bovine CRP Ab was purified by affinity chromatography. Pure bovine CRP (8 mg) previously prepared¹⁷⁾ was coupled to 5 ml Sepharose 4B (PHARMACIA LKB BIOTECHNOLOGY Inc., Piscataway, NJ, USA) according to the method described by March et al.⁶⁾. Goat anti-bovine CRP serum (60 ml) was dialyzed against 0.01 M Tris buffer (pH 8.0) containing 0.14 M NaCl and 0.01 M EDTA. The dialyzed sample was then applied to the CRP-conjugated Sepharose 4B column equilibrated with the same buffer and recycled twice at the flow rate of 10 ml/hr. After washing with 0.05% Tween-PBS, the bound protein was eluted with 3 M KSCN (pH 6.0). Fractions of the protein peak were pooled, dialyzed, concentrated and kept at -80°C until use. The purity and immunological activity of each Ab were confirmed by SDS-PAGE according to Laemmli⁵⁾ and Ouchterlony double immunodiffusion¹¹⁾ against bovine CRP, respectively (data not shown).

Coating of latex particles with purified Ab : Latex particles were separately coated with anti-bovine CRP IgG purified by DE-52 or affinity chromatography at various concentrations using a slightly modified method as described by Yamamoto et al.¹⁹⁾. Briefly, 2% Latex suspension in the glycine buffer was incubated with purified IgG at 0, 5, 10, 15, 20, 30 and 40 μg IgG/mg Latex at 37°C for 2 hr with shaking and then kept at 4°C

overnight. The pellet was obtained by centrifugation at 15,000 rpm. for 20 min., resuspended in 1% BSA in glycine buffer and incubated under the same conditions as described above. The pellet was then washed 3 times with the same glycine buffer containing 3% NaCl, 5% sucrose, 2% choline chloride and 0.02% NaN₃ and finally suspended at 1% concentration for use.

Latex agglutination test: Serum samples were serially diluted two-fold with 0.02 M Tris buffer (pH 7.4) containing 0.04 M NaCl and 1 mM EDTA. Then, 20 μ l of the diluted serum sample was mixed with 20 μ l of the glycine buffer containing 1% BSA and 20 μ l of 1% IgG coated latex on a clean glass slide. The mixture was incubated in a moist chamber for 45 mins. The agglutination was observed and compared with a standard serum of which the CRP concentration had been determined by SRID as shown in Fig 1. CRP concentration (conc) in the test serum was calculated as follows :

$$\text{CRP conc } (\mu\text{g/ml}) = A \times B$$

where, A=CRP conc in standard serum showing the same degree of agglutination as that seen with a diluted test serum

B=the dilution factor of the diluted test serum

Reproducibility of the assay: For the same-day assay, 4 serum samples with different CRP concentrations ranging from 15 μ g/ml to 170 μ g/ml were subjected to the latex agglutination test 5 times on the same day. The different-day assay was done by measuring CRP levels in those 3 serum samples 3 times a day for 5 consecutive days.

Analysis of the recovery assay: CRP levels were assayed in 3 sera with concentrations lower than 10 μ g/ml. The samples were supplemented with purified CRP at concentrations of 30, 60 and 100 μ g/ml and CRP concentrations in serum samples were measured by latex agglutination test.

Correlation of latex agglutination test with single radial immunodiffusion (SRID) test: CRP levels in 33 sera from clinically healthy dairy cows determined by latex agglutination test were compared to those measured by SRID test which were reported previously¹⁷). The correlation coefficient (r) was determined.

Effect of storage at 4°C and freezing-thawing on CRP: CRP concentration in 33 sera of clinically healthy dairy cows, which had been kept at 4°C for 2 years in the presence of 0.02% NaN₃ were determined by SRID. The results were compared to the levels of CRP in the same sera, measured by the same method, at the time of storage.

To study the effect of freezing and thawing on CRP, 10 bovine sera were used. EDTA was added to make a final concentration of 10 mM. The samples were then frozen at -20°C and subjected to freezing and thawing 5 times. Before freezing and after each thawing, CRP concentrations in serum samples were determined by SRID test.

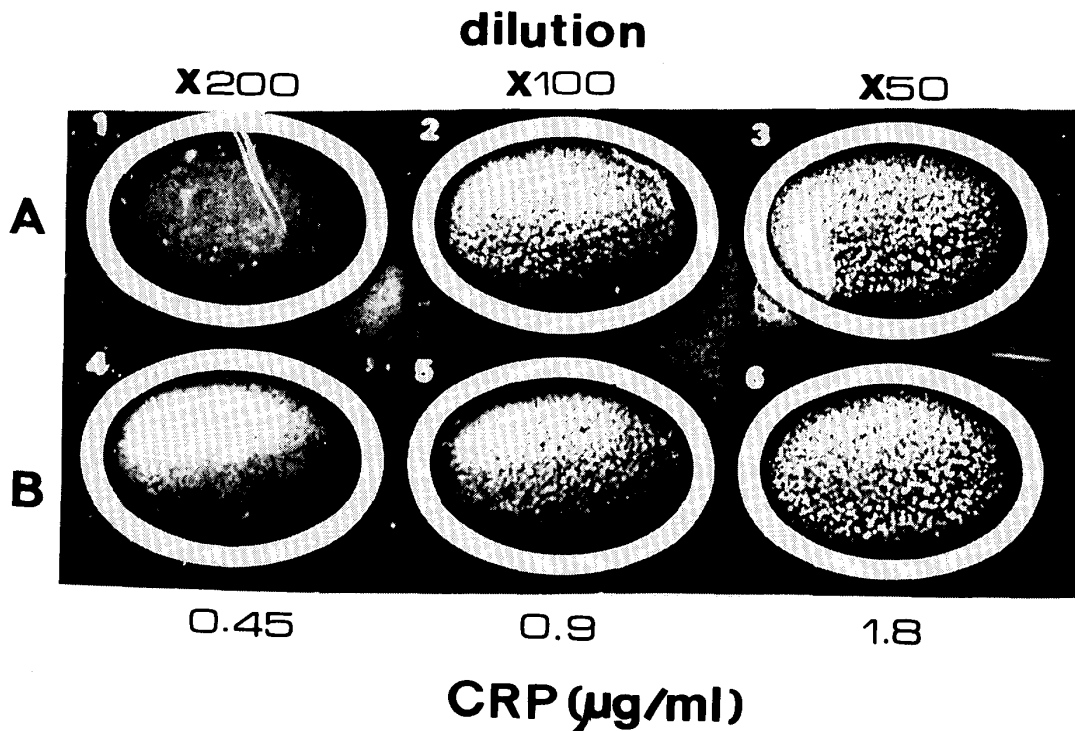


Fig 1. Latex agglutination test for determination of bovine serum CRP level was performed at room temperature in a moist chamber for 45 mins. In this case, test serum (A; the upper row) dilutions of 200 fold (1), 100 fold (2) and 50 fold (3) were used. The diluted standard sera (B; the lower row) containing 0.45 $\mu\text{g/ml}$ (4), 0.9 $\mu\text{g/ml}$ (5) and 1.8 $\mu\text{g/ml}$ (6) CRP concentration were used for comparison. CRP level in the test serum was calculated as follows:

$$\text{CRP conc.} = 1.8 \times 50 = \mu\text{g/ml}$$

RESULTS

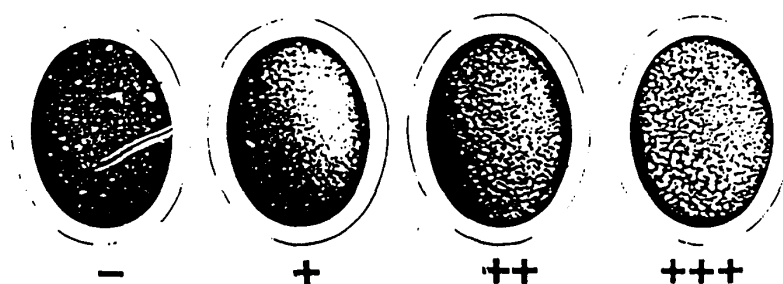
Table 1 shows the result of agglutination obtained when latex particles were coated with various concentrations of affinity-purified Ab. From the different amounts of Ab, 20 μg of Ab/mg latex was found to be the most suitable. The minimum CRP concentration detectable after the 45 min-incubation was 40 ng/ml (Table 2). When DE-52-purified Ab was used for coating, the optimal concentration for agglutination was 20 $\mu\text{g/mg}$ latex (data not shown).

At room temperature, the degree of agglutination changed with time. It was found that the optimal incubation time to produce a stable result was 45 mins (Table 2). The latex particles coated with affinity-purified Ab were observed to be more

Table 1. Evaluation of the optimal amount of antibody for coating the latex particles

| CRP concentration ($\mu\text{g}/\text{ml}$) | Affinity-purified Ab coated ($\mu\text{g}/\text{mg}$ latex) | | | | | | |
|--|---|---|----|-----|-----|-----|-----|
| | 0 | 5 | 10 | 15 | 20 | 30 | 40 |
| 0.030 | — | — | — | ± | ± | — | — |
| 0.060 | — | — | — | + | + | — | — |
| 0.125 | — | — | + | + | + | + | — |
| 0.25 | — | — | + | + | + | + | + |
| 0.50 | — | + | + | + | + | + | + |
| 1 | — | + | + | + | + | + | + |
| 2 | — | + | + | + | + | + | + |
| 4 | — | + | ++ | + | ++ | ++ | ++ |
| 8 | — | + | ++ | ++ | ++ | ++ | ++ |
| 16 | — | + | ++ | ++ | ++ | +++ | +++ |
| 30 | — | + | ++ | +++ | ++ | +++ | +++ |
| 50 | — | + | ++ | +++ | +++ | +++ | +++ |
| 100 | — | + | ++ | ++ | +++ | +++ | +++ |
| 200 | — | — | + | ++ | +++ | +++ | ++ |
| 400 | — | — | + | ++ | +++ | ++ | + |
| 800 | — | — | + | ++ | + | + | + |
| 1600 | — | — | + | + | + | + | + |

Latex particles were coated with various amounts of affinity-purified antibody and subjected to the agglutination test using bovine CRP at various concentrations. Negative (—), weak positive (\pm) and positive (+ to +++) grades of agglutination were indicated. Degrees of agglutination are shown in the following photograph.



sensitive in detecting lower concentrations of CRP than those coated with DE-52-purified Ab (Table 2). Thus, subsequent experiments were carried out using latex particles coated with affinity-purified Ab at a concentration of 20 $\mu\text{g}/\text{mg}$ latex. To determine the level of CRP in serum, a semi-quantitative latex agglutination test was developed as shown in Fig 1.

Table 3 shows that both the same-day assay and different-day assay gave a

Table 2. Incubation time and detection limit of bovine CRP by the latex agglutination test using 2 antibodies obtained by different purification procedures

| Incubation time (min) | Detectable CRP concentration ng/ml | |
|--------------------------|---------------------------------------|-------------------------|
| | DE-52-purified Ab | Affinity-purified Ab |
| 10 | 2720 | 680 |
| 15 | 1360 | 680 |
| 20 | 1360 | 80 |
| 30 | 340 | 80 |
| 45 | 340 | 40 |

Table 3. Reproducibility of the assay

| CRP concentration, $\mu\text{g}/\text{ml}$ | | CV ^a % |
|--|--------|-------------------|
| A. Same-day assay ^b | | |
| mean | (SE) | |
| 14.7 | (1.2) | 15.6 |
| 31.8 | (0) | 0 |
| 97.5 | (7.5) | 17.1 |
| 169.5 | (10.5) | 13.9 |
| B. Different-day assay ^c | | |
| mean | (SE) | |
| 31.8 | (0) | 0 |
| 90 | (0) | 0 |
| 162.3 | (9.5) | 17.9 |

^a Coefficient of variance.

^b Five assays repeated within the same day.

^c Assays repeated 3 times a day and on 5 consecutive days.

maximum coefficient of variance (CV) of less than 20%. The results of analysis of the recovery assay (Table 4) revealed that 85–95% of added CRP can be detected by this method. The latex agglutination test was found to have correlation with the SRID test as indicated by a correlation coefficient (r) of 0.95 (Fig 2). Fig 3 shows that CRP concentrations decreased by about 30% ($p < 0.001$) compared to those in fresh sera. Addition of EDTA to the sera was found to prevent the decrease of serum CRP concentration after freezing and thawing (Table 5).

Table 4. Analysis of recovery of CRP in the assay

| Added CRP $\mu\text{g/ml}$ | Recovered CRP mean (SE) $\mu\text{g/ml}$ | Recovery (%) mean (SE) |
|-------------------------------|--|---------------------------|
| 30 | 28.7 (3.1) | 95.7 (10.4) |
| 60 | 51.2 (6.2) | 85.4 (10.4) |
| 100 | 90 (0) | 90 (0) |

$n = 3$ each

Different amounts of CRP were added to sera containing CRP of less than $10\mu\text{g/ml}$ and then CRP concentrations were determined by latex agglutination test.

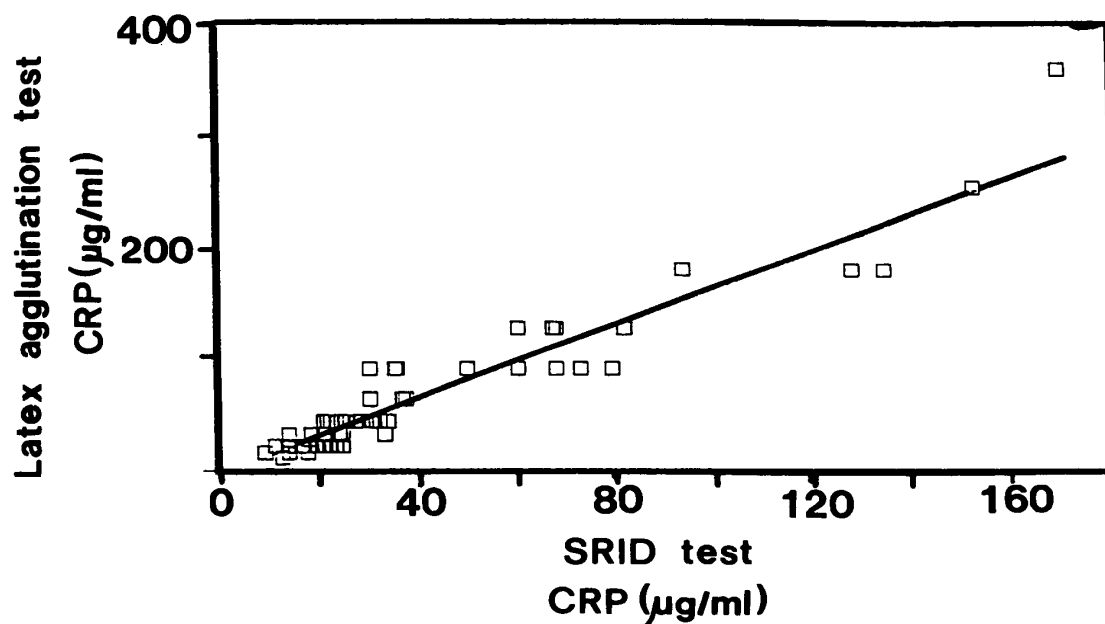


Fig 2. Correlation between the latex agglutination test and SRID test for bovine CRP determination. The correlation coefficient (r) was calculated to be 0.95.

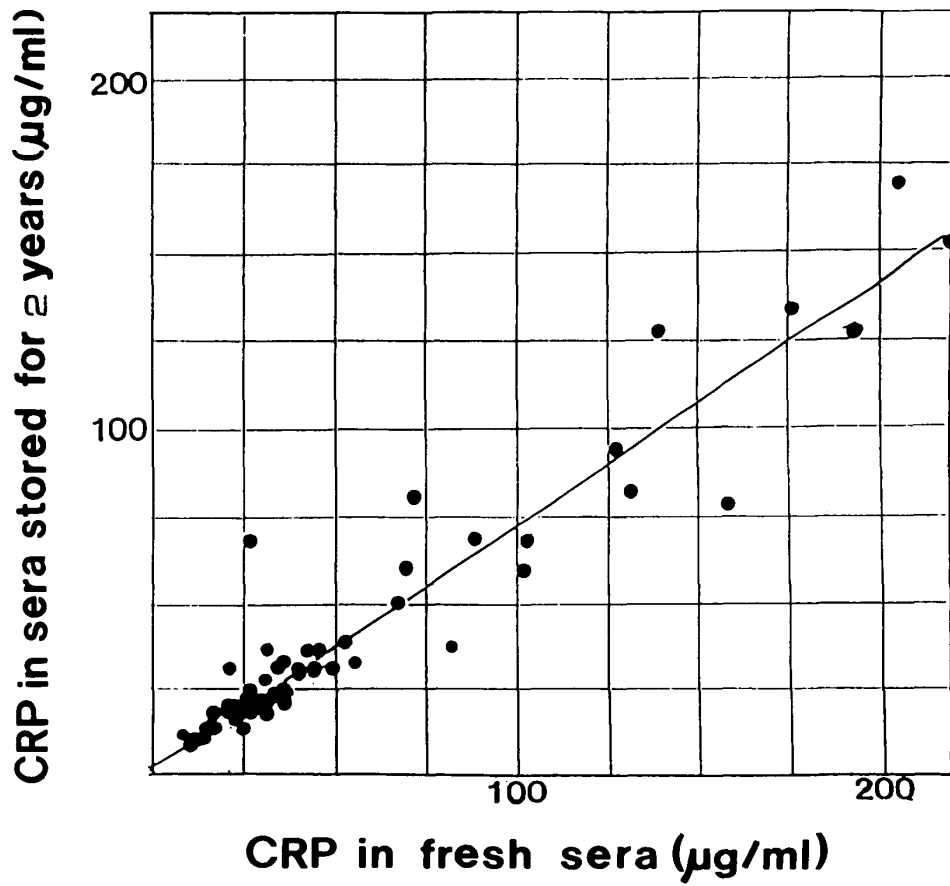


Fig 3. Effect of 2-years of storage at 4°C on bovine CRP level. CRP concentrations in 33 sera were determined by SRID.

Table 5. Protective effect of 10mM EDTA addition against a reduction of serum CRP concentration in freezing-thawing.

| | CRP concentration ($\mu\text{g}/\text{ml}$) freezing-thawing | |
|------|---|-------|
| | Before | After |
| | 170 | 179 |
| | 135 | 113 |
| | 128 | 144 |
| | 128 | 109 |
| | 94 | 97 |
| | 79 | 78 |
| | 73 | 57 |
| | 68 | 62 |
| | 60 | 75 |
| | 60 | 47 |
| mean | 99.5 | 96.1 |

10mM EDTA was added to 10 different sera and freezing-thawing at -20°C was repeated 5 times and serum CRP concentrations were determined by SRID.

DISCUSSION

The latex coating procedure described in this paper was straightforward because antibodies such as IgG are known to be adsorbed readily to polystyrene latex³). The presence of NaCl, sucrose and choline chloride in the storage buffer provides an increase in density of the latex suspension and specific agglutination due to a corresponding reduction of non-specific agglutination. Absence of spontaneous agglutination is considered as one of the criteria in establishing suitable conditions for the latex agglutination test presented in this paper. The latex particle itself contains specific charges on its surface and these can be affected by the protein coat. Thus, a certain size of latex particle coated with an inappropriate amount of protein, or vice versa, can generate unpredictable changes in its surface charges, resulting in self-agglutination in the absence of antigen. Preliminary tests revealed that the presence of a non-reactive protein such as BSA is required for agglutination. The mechanism was not quite understood but it was found that 1% BSA in reaction buffer provides a better agglutination than 0.5% or 0.1% BSA.

In experiments, when determining the optimal amount of Ab for coating latex particles, 15 μg of Ab/mg latex and 20 μg of Ab/mg latex were found to give similar

results. However, 20 μg of Ab/mg latex was chosen as the most suitable amount of Ab to coat the latex particles in subsequent tests. This is because 20 μg of Ab/mg latex showed a higher degree of agglutination at high CRP concentrations of 100–200 $\mu\text{g}/\text{ml}$, which are usual in the serum of cattle with an acute-phase response. The latex particles coated with affinity-purified Ab could detect CRP at a concentration 8 times lower than those coated with DE-52-purified Ab at the same concentration. This might be attributed to a higher proportion or purity of specific Ab in the affinity-purified preparation.

The reproducibility of this test was found to be acceptable because the maximal CVs of the same-day and different-day assay were 17% and 18%, respectively. The accuracy of the latex agglutination test, evaluated through analysis of the recovery assay, was sufficient with a recovery of 85–95%. Correlation analysis between the present method and SRID test showed a correlation coefficient (r) of 0.95 which confirms the accuracy of the method described here.

In previous studies, there was evidence showing that freezing broke down serum CRP (Yamamoto, S., Azabu College of Veterinary Medicine, personal communication). In this paper, the effect of long term storage at low temperatures on CRP concentration was investigated. The fall in CRP concentration was about 30% over a 2-year-storage period. This is probably due to spontaneous denaturation of serum lipoprotein which is known to bind CRP in the presence of the calcium ion¹⁵). Subsequently, the denatured lipoprotein might generate a partial denaturation of some bound CRP molecules, which causes conformational change in CRP, resulting in it being undetectable in the immunological assay (SRID). In addition, it might also be attributed to a partial degradation by protease enzymes naturally found in the serum. The degradation effect of freezing (at -20°C) on very low density lipoprotein (VLDL) and low density lipoprotein (LDL) is already known. EDTA can inhibit most of the CRP-lipoprotein interaction and 10 mM EDTA seems to be enough to generate a complete dissociation of the CRP-lipoprotein complexes. Since the effective concentration of EDTA to inhibit metalloprotease enzymes is usually 0.5–2 mM, 10 mM EDTA can overcome the effect of such enzymes, if present, in the serum. In addition, the activities of other serum protease enzymes are decreased at temperatures below freezing (-20°C) and seem unlikely to be involved in the decrease of serum CRP level after repeated freezing and thawing. However, further study of the role of EDTA in lipoprotein-depleted serum is needed to provide an explanation for the effect of freezing on CRP level.

In cattle, CRP also acts as an acute-phase protein and the measurement of serum CRP concentration can provide information about the degree of inflammation (Morimatsu et al., unpublished data). In large animal clinics, the use of detection methods such as ELISA or SRID, is time-consuming and / or requires special equipment. The latex agglutination test, although a semiquantitative measurement, overcomes these

problems, being rapid and simple. Moreover, it can give a reliable and reproducible result as well as a good correlation with the conventional SRID test.

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