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Serum, plasma, and dried blood spot high sensitivity C-reactive protein enzyme immunoassay for population research

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Abstract

C-reactive protein (CRP) is used as a biomarker of morbidity and mortality risk in studies of population health, and is essential to interpretation of several micronutrient biomarkers. There is thus need for a robust high sensitivity CRP (hsCRP) measurement method for large-scale, non-clinical studies. We developed an efficient, inexpensive assay suitable for quantifying CRP across the physiological range using any blood specimen type. The ELISA uses readily available monoclonal antibodies to measure CRP in serum, plasma, or dried blood spots (DBS) made from venous or capillary blood. Assay performance was evaluated by standard methods, including comparison with a previously described assay. Effects of specimen type were tested by measuring CRP in 52 matched serum, plasma, and venous and capillary dried blood spot specimens. Long- and short-term CRP stability were evaluated. Assessments of assay limits of detection, linearity, recovery, imprecision, and concordance with an established method (Pearson correlation = 0.988, $n = 20$) demonstrated the validity of the new assay. CRP measurements in serum, plasma, and DBS had Pearson correlations from 0.974 to 0.995, $n = 52$, but CRP in serum was on average 1.6 times (SD 0.37) higher than in DBS. CRP was stable in frozen serum for up to 34 months, but DBS CRP declined quickly with exposure to ambient temperatures, and across long-term storage at -20°C . This hsCRP assay is a robust and inexpensive tool designed for use in large-scale population health research. Our results indicate that DBS CRP is less stable than previously reported.

Keywords

Blood spots; Stability; C-reactive protein; inflammatory marker

1. Introduction

C-reactive protein (CRP) is a widely-used marker of both chronic and acute inflammation (Pepys and Hirschfield, 2003), with great utility in interpretation of micronutrient biomarker measurements affected by active infection (Thurnham et al., 2005; Kung'u et al., 2009).

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High-sensitivity CRP (hsCRP) assays have revealed its importance as an indicator of morbidity and mortality risk (Danesh et al., 2000; Ford et al., 2001; Pearson et al., 2003; Finch and Crimmins, 2004; Ridker et al., 2004; Ridker et al., 2008). Several methods for measuring hsCRP are available (Roberts et al., 2001; Clarke et al., 2005), including those designed for clinical diagnostic use and commercial assay kits designed for non-clinical research. However, these can be prohibitively expensive (more than \$5 per specimen) for large-scale research, may be limited in the specimen types demonstrated to yield valid results, and may not be efficient for high-throughput. An in-house assay described by Wu et al. (2002) for use in serum, and later by McDade et al. (2004) for use in dried blood spots (DBS), effectively filled the demand for less expensive and more efficient assays suitable for large-scale population research with limited budgets and facilities, but the polyclonal antibodies needed are no longer available. Our goal was to simplify and streamline population research by creating an ELISA method using basic equipment and commercially available monoclonal antibodies (MAb) from an established supplier.

While serum or plasma specimens have been the gold standard for CRP measurements, demands of collecting, processing and storing these specimen types can be obstacles in non-clinical settings or remote field sites. DBS have been gaining interest as a more robust and convenient alternative (McDade et al., 2007; Skogstrand et al., 2008). We sought an assay useful for measuring CRP in serum, plasma, and DBS made using either capillary or venous blood collection methods.

We report here on a new, high-sensitivity CRP ELISA that is designed to be efficient and inexpensive (\$0.50 or less per specimen) for use in large-scale population health research. In addition, we report results of tests designed to determine stability of CRP in serum or DBS under the less than ideal storage conditions that are often encountered in non-clinical research.

2. Materials and Methods

2.1 Specimens

A summary of all specimens used for evaluating CRP assay performance and stability of CRP is given in Table 1. Research procedures for all specimen collections were approved by the Institutional Review Boards of the University of Washington and/or the Kenya Medical Research Institute (KEMRI).

For evaluating assay performance across sample types, 52 matched sets of specimens of four types (serum, plasma, capillary DBS collected from a finger prick and DBS prepared from venous blood collection) were collected simultaneously from US men and women, ages 25–50. Blood was collected by venipuncture into tubes containing K₂-EDTA anticoagulant and tubes without additives. Plasma was separated from the cells within 1 h of collection; serum tubes were allowed to clot 1 h at room temperature, and then centrifuged to separate serum samples. A second tube of venous blood collected without additives was immediately spotted onto filter paper from a wide-bore pipette tip to prepare venous DBS. Capillary DBS were collected by finger prick with a sterile safety lancet. The first drop of blood was wiped away, and then blood was allowed to fall freely onto filter paper. Venous and capillary DBS were prepared using Whatman 903 Protein Saver cards, dried thoroughly 3–5 h at room temperature in a climate-controlled lab, and stored in sealed plastic bags with desiccant packets. All specimen types were stored frozen at –20°C.

2.2 CRP Stability Tests

In addition to the matched specimens used to assess assay performance by sample type, three distinct sets of specimens were used to assess stability of CRP: 1) capillary DBS

specimens collected in the lab for short-term stability tests; 2) matched serum and DBS collected in the lab for long-term stability tests; and 3) matched serum and DBS collected in rural Kenya used to evaluate a combination of short and long term storage conditions following collection in a remote field setting.

The first set of specimens, a convenience sample of 8 capillary DBS specimens, were collected in the lab from US adults by finger prick to test short-term DBS stability. Specimens were subjected to conditions designed to simulate several lab and field sample treatment scenarios. All DBS were stored frozen (-20°C) in air-tight containers with desiccant packets for up to 3 wk while sample collection was underway, then assayed in a single batch to establish a baseline. DBS were tested after storage at 22°C (controlled ambient), 37°C (hot ambient), and -20°C (frozen) for 7, 14, 28 and 42 d. To assess the effects of temperature fluctuation, we subjected DBS to 1, 2, 4 and 8 cycles of freeze-thaw (22°C for 8 h then -20°C for 16 h) or hot-cool (37°C for 14 h then 17°C for 10 h); specimens were stored at -20°C for 1 to 14 d after completion of the assigned number of cycles, then assayed in a single batch. Temperature was recorded throughout these experiments to assess degree of variation from the intended storage conditions.

A second set of specimens, a convenience sample of matched serum and venous DBS collected in the lab from 8 US adults for another study, were assayed after 161, 374, 764 and 1026 days of storage at -20°C to assess long-term stability of CRP in frozen specimens. Day 161 results were treated as the baseline, and compared to all subsequent assay results.

The third set of specimens, matched venous DBS and serum from 23 women randomly selected from a larger study, were field-collected, and reflect a real-world combination of short-term and long term field treatment effects. These were collected as part of a study of 241 lactating Ariaal women of Marsabit District, northern Kenya (Fujita et al., 2009), originally with no intent to use the specimens to investigate CRP stability. Venous blood collected without additives was immediately spotted onto filter paper by pipette. After drying, DBS were stored in a sealed plastic bag with desiccant at ambient field temperatures ($12\text{--}28^{\circ}\text{C}$) for 13–42 d. Serum was separated by centrifuge attached to a car battery and within 3 h of venipuncture frozen and stored in liquid nitrogen for 13–42 d before transport to the KEMRI laboratory in Nairobi, where they were stored frozen at -20°C . All samples were then shipped via air express on dry ice to UW and stored at -20°C until assay 8 mo after collection.

2.3 Assay Methods

Capture (clone C5 anti-CRP MAb, cat.no. M86005M) and detection (clone C6 anti-CRP MAb, cat.no. M86284M) antibodies recommended as a matched pair were purchased from Biodesign International. The detection antibody was conjugated to biotin following the method of Hermanson (1996). One mole of antibody to 12 moles of NHS-LC-biotin ester (Sigma-Aldrich) dissolved in dimethylformamide were combined in pH 7.2 phosphate buffered saline, and allowed to react at room temperature with stirring for 1 h. Conjugated antibody was separated from free excess biotin by dialysis against phosphate buffered saline, pH 7.2, for 48 h at 4°C . Biotinylated C6 anti-CRP MAb was aliquoted and stored at -80°C . Both the capture and conjugated detection antibody are expected to tolerate storage for several years without loss of activity (Harlow and Lane, 1999).

DBS specimens were eluted from filter paper by immersing one 1/8" punch (equivalent to $1.525\mu\text{L}$ serum (Mei et al., 2001)) in a volume of assay buffer (0.25 to 5.00 mL; 0.01M PBS, 0.5M NaCl, 0.1% v/v Tween 20, pH 7.2) needed to achieve the desired dilution, generally 1:200 to 1:400. All punches were taken from the periphery of the blood spots to minimize effects of small differences in serum volume ($<2\%$) with the spread of blood

across the spot (Mei et al. 2001). After overnight incubation at 4°C, specimens were allowed to shake for 30–60 min before eluates were separated from the filter paper and added to the plates the same day. Serum and plasma were likewise assayed at dilutions of 1:200 to 1:400 in assay buffer, prepared on the same day they were added to the plates.

Microtiter plates (Nunc, cat.no. 442404) were coated with 100µL of 1µg/mL anti-CRP MAb clone C5 diluted in coating buffer (0.20 M NaHCO₃, pH 9.6), and incubated overnight or up to 3 d at 4°C. Plates were washed (0.15 M NaCl; 0.05% Tween 20), then blocked for 30 min with 200µL per well of assay buffer. Plates were washed again, and 100µL of serum, plasma, or eluted DBS specimens, quality control specimens, and calibrators diluted in assay buffer were added in duplicate to the wells. All specimen types with results outside the calibration range were repeated with dilutions adjusted as needed, from 1:50 for very low CRP specimens up to 1:3200 for those with high CRP.

An 8-point calibration curve made from purified human CRP (Fitzgerald Industries International, Inc., cat.no. 30-AC10) was run on every plate (0, 0.0003, 0.0006, 0.0013, 0.0025, 0.0050, 0.0101, and 0.0202 mg/L). For serum and plasma specimens, calibrators were made fresh for each assay from concentrated liquid stock, stored aliquoted and frozen following the manufacturer's instructions. For DBS, calibrator was combined with washed erythrocytes and preserved on filter paper following the method described by McDade et al. (2004) at concentrations yielding the same calibration curve range when 1/8" punches from the spot were eluted in 2mL assay buffer overnight at 4°C. Fresh calibrators were prepared for each assay batch.

After overnight incubation at 4°C, the plates were washed, and 100µL/well of 500ng/mL biotinylated detection MAb C6 in assay buffer was added to the plate. After incubation for 2 h at room temperature, plates were washed and 100µL/well of horseradish peroxidase streptavidin (Invitrogen Corporation) diluted 1:6000 in assay buffer was added. After incubating 1 h at room temperature, plates were washed again, and developed in citrate buffer (50 mmol citrate, pH 4.0) combined with 0.4 mmol 2,2-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (Sigma-Aldrich) and 1.6 mmol of hydrogen peroxide (100µL/well). Color was allowed to develop on an orbital plate shaker for 45 min – 1 h before plates were read (test 405nm, ref 570nm) with a microtiter plate reader (Synergy HT, BioTek Instruments, Inc.) and concentrations were estimated with a four-parameter logistic calibration curve (Gen5, BioTek Instruments Inc.).

2.4 Assay Validation

Controls were run on every plate. Plasma controls were used for plates with serum and/or plasma specimens, and DBS controls were used with DBS specimens. Plasma controls were collected from a healthy US adult and diluted to three concentrations. DBS controls were prepared from 3 venous blood specimens dropped onto filter paper, 50µl per spot, using a wide-bore pipette tip. Assay imprecision was estimated using a variance components model (Rodbard, 1974).

Analytical sensitivity was defined as the concentration 3 SD above the zero dose calibrator (Rodbard, 1978; O'Fegan, 2000). Functional sensitivity was estimated by calculating a precision profile (n=230 samples), and was defined as the concentration at which the within-assay CV is consistently less than 10% (Davies, 2005). Independence of volume was tested using ten serially diluted plasma specimens and ten serially diluted venous DBS specimens.

Recovery of CRP from a plasma matrix was estimated by spiking six plasma specimens with low, medium, and high doses of purified human CRP. Plasma specimens were selected to include low, medium, and high endogenous CRP, and were diluted 1:400 or 1:800 in assay

buffer before adding measured doses of CRP prepared in assay buffer. Varying doses were added as 10% of the total sample volume to maintain consistency from dose to dose, and spiked specimens were run in triplicate in each of 10 independent assay batches. Mean $\pm$ SD recovery was calculated as [observed CRP value / expected CRP given the concentration native to the sample plus the known added dose of calibrator] (O'Fegan, 2000)

To assess specificity, two compounds related to CRP, human pentraxin 2 and human pentraxin 3 (Garlanda et al., 2005), were added to the wells at 0, 0.020, 0.202, 2.021, and 20.206 mg/L in assay buffer. In addition, a diluted plasma specimen was spiked as above to test for negative assay interference resulting from cross-reactivity (Davies, 2005). Results were calculated as percent of endogenous CRP values recovered.

Results of the assay described here using antibodies from Biodesign were compared with the method described by McDade et al. (2004) using Dako antibodies (cat.no. A0073 coating antibody and P0227 detection antibody, both now discontinued). CRP was measured in 4 capillary DBS specimens run at 5 dilutions each ($n = 20$ specimen-dilution combinations) to test the full range of the calibration curve for both assays. Lack of available supplies limited the sample size for this comparison.

2.5 Statistical Methods

Linear mixed effects models were used to test for differences between slopes of serially diluted specimens and the calibration curve and to test whether dilution-corrected slopes differed from zero, with random effects estimated for the intercepts and slopes. CRP measurements from serum, plasma, capillary DBS and venous DBS specimens ($n = 52$ matched sets) were compared by Pearson correlation, and absolute levels were compared by linear regression, Bland-Altman analysis (Bland and Altman, 1999) and by expressing results from plasma and both types of DBS as a percentage of the serum results. Results for the same DBS specimens ($n=20$) assayed for CRP by our new assay and a previously published method (McDade et al., 2004) were compared by Pearson correlation, paired t-test, and percent agreement.

Both short term laboratory stability for DBS CRP ($n = 8$ specimens per treatment) and long term stability DBS and serum CRP ($n = 8$ matched specimens per sample type) were analyzed using linear mixed effects models. This approach separates between-specimen variability from within-specimen variability, and is appropriate for use with repeated measures of the same specimen over time, where measurement independence can not be assumed. Random effects were estimated to account for across subject variability in the magnitude of baseline CRP. Average log CRP linear regression lines by time or cycle were estimated with random effects estimated for both intercepts and slopes. Log CRP was also modeled as categorical to assess non-linear trends. For long term stability, DBS and serum CRP were analyzed separately. Linear mixed effects models were also used to estimate average DBS and serum log CRP at each long term storage time point; plots of average CRP were constructed by storage time. Effects of serum and DBS ($n = 23$ matched sets) collection and ambient temperature DBS storage under remote field conditions were examined by comparing the relationship between frozen serum CRP and DBS CRP by days of DBS exposure to ambient temperatures.

Linear mixed effects models were carried out using R 2.9.0 (R Development Core Team 2009). For all tests, $P < 0.05$ was considered significant.

3. Results

3.1 Assay Performance

For plasma controls, within and between assay CVs were 3.5% and 9.4% respectively for the low (0.0033mg/L) control, and 2.7% and 9.9% for the high (0.0063mg/L) control (n=11 plates). For DBS, within and between assay CVs were 3.4% and 9.9%, respectively, for the low (0.0017mg/L) control and 2.4% and 3.4% for the high (0.0045mg/L) control (n=20 plates).

Analytical sensitivity was 0.00007mg/L (n=20 plates), while functional sensitivity was 0.00015mg/L (n=230 specimens, run in duplicate, across 8 plates). Mean $\pm$ SD recovery was 105% $\pm$ 4%, 101% $\pm$ 2%, and 106% $\pm$ 5% for low, medium, and high doses respectively.

Assay linearity was tested across a broad range of concentrations (raw values, 0.0003 to 0.0141 mg/L; dilution adjusted values 0.026 to 3.633 mg/L) and dilution ranges (1:50 to 1:6400) (Fig. 1). Linear mixed effects models showed no difference between slopes of the liquid calibration curve and of ten serially diluted plasma specimens (p=.9), or between slopes of the DBS calibration curve and ten venous DBS specimens serially diluted after elution (p=.4). When concentrations were multiplied by dilution factor, linear mixed effects models showed a slight, but not significant, negative average slope for plasma specimens (-0.03 ± 0.02 , p=.11), and a slight and significant negative average slope for DBS specimens (-0.07 ± 0.02 , p=.0011). CV calculated across dilutions (n=3 to 6 dilutions per specimen, mean=5 dilutions per specimen) to assess variability introduced by the negative slopes averaged 9% $\pm$ 3% for plasma and 10% $\pm$ 5% for DBS, indicating the differences in concentration between the lowest and highest dilutions are within the range of assay imprecision estimates.

Tests for assay cross-reactivity with related compounds found that neither human pentraxin 2 nor human pentraxin 3 gave detectable results at any dose. In the test for negative assay interference from human pentraxin 2 or human pentraxin 3, no interference was evident from either analyte at physiological levels. Slight negative interference was detected from pentraxin 3 at 20.2 mg/L (10,000 times normal physiological values, and 1,000 times the values reported in diseased state (Peri et al., 2000)), with mean $\pm$ SD recovered concentrations of 95% $\pm$ 1%, 98% $\pm$ 4%, 95% $\pm$ 1%, and 98% $\pm$ 7% for pentraxin 2 and 96% $\pm$ 2%, 97% $\pm$ 3%, 95% $\pm$ 4%, and 88% $\pm$ 5% for pentraxin 3 at 0.020, 0.202, 2.02, and 20.2 mg/L, respectively.

CRP measurements from serum, plasma, and both types of DBS specimens (n=52 specimens per sample type) were significantly correlated, with Pearson correlations ranging from 0.974 to 0.995 (Table 2). Absolute values of CRP in plasma specimens were on average 93% $\pm$ SD 16% of serum CRP concentrations. There was also agreement between capillary and venous DBS CRP results, with venous DBS results averaging 99% $\pm$ 15% of capillary DBS CRP values. Both capillary and venous DBS gave results lower than serum or plasma CRP concentrations: capillary DBS concentrations were 69% $\pm$ 18% of serum CRP, and venous DBS were 67% $\pm$ 15% of serum CRP. These differences in absolute value are evident in linear regression plots (Fig. 2). Bland-Altman analysis shows the magnitude of the differences between serum and plasma, and between serum and DBS of both types; differences were consistent across concentration (Fig. 3). The mean (SD) ratio of DBS (venous or capillary) to serum was 1.6 (0.37).

Capillary DBS results from a previously published DBS CRP method (McDade et al., 2004) and our new assay were significantly correlated: Pearson correlation was 0.989 (n=20), and absolute values did not differ significantly (p=0.843), with the previous assay's results

averaging $95\% \pm 9\%$ ($n=20$ specimens) of CRP results obtained using the new assay described here.

3.2 CRP Stability

Controlled short-term stability experiment results are shown in Table 3. Means are parameter estimates from unlogged data, while hypothesis testing was carried out using logged CRP. Recorded temperatures during the treatment regimen deviated 5% or less from intended experimental conditions. Sample volume and experimental design did not allow for adjusting dilution and repeating assays for samples that fell below the lower limit of detection. In order to avoid potential bias introduced by leaving out results for these samples, results too low to be quantified as assayed were assigned a value just below the detectable limit given the dilution used. Excluding these samples from the analysis did not alter the results. In linear mixed effects analysis, there was no significant decline ($p=.6$) in DBS CRP up to 42 days of storage at -20°C , and a borderline significant effect ($p=.013$) of up to 8 freeze-thaw cycles. Most of this significance was due to a larger average decline at the last cycle. Excluding the last cycle from the model, there was no significant decline in DBS CRP ($p=.2$). The model detected significant degradation of CRP in DBS stored at cool ambient temperatures (22°C) for 14 days or more, and in DBS stored at hot ambient temperatures (37°C) for 7 days or more. DBS subjected to temperature cycles simulating daily fluctuations in field temperature conditions ($17^{\circ}\text{C}/37^{\circ}\text{C}$) showed degradation very quickly: there was a significant decline ($p=.0029$) in CRP after only 12 hours storage at 37°C followed by 12 hours at 15°C .

CRP results from serum and DBS collected in the laboratory, and assayed after 161, 374, 764 and 1026 days of storage at -20°C are shown in Fig. 4. DBS CRP declined when modeled linearly ($p<.0001$) by an average of 0.004 log mg/L per 100 days. Taking the anti-log of the model estimates, at day 161, DBS CRP was 0.86 mg/L and at day 1026 it was 0.62 mg/L. Serum CRP did not demonstrate an overall linear decline (slope=0.0007 log mg/L per 100 days, $p=.4$).

Serum and DBS specimens stored in rural Kenya field conditions, then stored for almost 12 mo at -20°C in the laboratory, showed evidence of CRP degradation in DBS related to duration of storage at ambient temperatures (Fig. 5). Pearson correlations between serum and DBS were significant (.913, $p<.001$) and DBS values averaged $61\% \pm 20\%$ of serum values, consistent with differences seen in lab-collected matched specimens. However, there was a negative correlation between the ratio of DBS CRP to serum CRP and time DBS spent stored at ambient temperature in the field (Fig. 4, Pearson correlation = $-.469$, $p=.024$). CRP in DBS specimens stored ≤ 20 d in the field before frozen storage ($n=9$) averaged $76\% \pm \text{SD of } 17\%$ of serum CRP, while DBS stored ≥ 21 d at ambient field temperatures ($n=14$) averaged $51\% \pm 15\%$ of serum CRP ($p=.001$). These results are consistent with the results of the stability tests in a controlled lab setting, in which DBS stored at room temperature were significantly different from baseline at 28 d, but not at 14 d, while DBS and serum CRP values were unchanged after a year of storage at -20°C .

4. Discussion

This hsCRP assay is suitable for use with serum, plasma, or DBS prepared from capillary or venous blood. Results did not differ between serum and plasma specimens, or between DBS specimens prepared from capillary versus venous blood. We did, however, find that DBS values were consistently lower than serum or plasma values. Similar differences in serum or plasma versus DBS levels have been reported previously for CRP and other analytes ((McDade and Shell-Duncan, 2002; McDade et al., 2004; Miller et al., 2006; Baingana et al., 2008) and others). These differences might stem from inaccuracy in the estimate of sample

volume contained in DBS punches (Mei et al., 2001), or might indicate incomplete elution of the CRP from the paper. Because the differences in CRP between DBS and serum were reasonably consistent, adjustment of DBS data to serum equivalents, or adjustment of cut-off values derived in serum for use with DBS, can easily accommodate observed differences in absolute value.

Protection from hot ambient (37°C) temperatures is clearly necessary. However, with proper protection from extreme heat, stability of CRP in DBS may be adequate to allow storage at controlled ambient temperature (around 22°C) for up to a week before freezing specimens. The results of our temperature fluctuation tests indicate that DBS CRP will tolerate repeated short intervals (8 h) of storage at moderate ambient temperatures, and repeated freeze thaw cycles. This would make DBS a practical alternative to serum or plasma, which require immediate processing with a centrifuge and continuous cold storage, which can be logistically difficult in remote field conditions.

Our DBS CRP short-term stability results are suggestive of some important differences from those in McDade et al. (2004) and Skogstrand et al. (2008). McDade et al. reported that DBS were stable for up to 3 d at 37°C and up to 3 d of cycling between 32°C and 22°C, and Skogstrand et al. find no effect of up to 30 d room temperature storage and up to 7 d storage at 35°C. In our tests of temperature fluctuations, in which specimens were stored first for 14 hours at 37°C, then for 10 hours at 17°C, we found significant degradation after only one cycle. These discrepancies may result from differences in the design of the experiments, and methods used to evaluate the results. Sample sizes were similar for the two studies (7 specimens for the present study versus 9 samples for McDade et al.). The average of baseline CRP values was higher in results reported by McDade et al., but the range of baseline values overlapped: the nine specimens used by McDade et al. averaged 3.48 mg/L, with a minimum of 1.11 mg/L and a maximum of 6.64 mg/L, while the seven specimens used in our experiments had average, minimum and maximum baseline CRP values of 1.92 mg/L, 0.23 mg/L and 7.33 mg/L, respectively. Erhardt et al. (2007) tested DBS CRP stability in similar, but not identical conditions, and reported results in agreement with those presented here.

Our results from field-collected paired serum and DBS samples showed evidence of degradation for samples stored in the field for more than 20 d at ambient temperatures. These data were not collected with the intent of investigating CRP stability in DBS, and as such represent use of cross-sectional data to address a question better answered by repeated measures over time. Our evidence of CRP degradation in DBS relies on changes in the relationship between DBS and serum CRP measures, and therefore carries the risk of spurious correlation (Kronmal, 1993). However, these specimens offer the advantage of giving insight into the effects of real, rather than simulated, field storage conditions. Although our sample size of field-collected specimens is small, and they do not represent a controlled CRP stability test, the results strongly suggest that care should be taken to protect DBS from warm ambient field temperatures. These data are also suggestive that the degree of variability that can be introduced by inconsistent storage conditions across specimens can be substantial.

Although we did not explicitly test the stability of our CRP calibrators, in our experience there is evidence to suggest degradation occurs in DBS, but not liquid, after about one year of storage at -20°C (data not shown). DBS calibrators should thus be stored protected from humidity at or below -20°C, monitored carefully for signs of deterioration, and made fresh at least once a year.

Use of these antibodies to measure CRP using a different protocol was described in brief by Skogstrand et al. (2008), but no evaluation of assay performance characteristics was reported. The protocol used here differs from that described in McDade et al. (2004), in that assay steps are carried out over three days instead of two. This format is intended to be more efficient for large-scale work, as it makes assay of larger batches of specimens practical. However, modifications to the protocol to shorten the total time needed to complete the assay may be possible.

Practical considerations, including cost (about \$0.50 per specimen for assay supplies versus \$5.00 – \$15.00 for commercial kits) and efficiency, and the performance characteristics described here support this CRP assay as a useful tool for population research.

Abbreviations used

CRP	c-reactive protein
hsCRP	high sensitivity c-reactive protein
DBS	dried blood spots
MAB	monoclonal antibody

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Figure 1.
Assay linearity for plasma (panel A) and venous DBS (panel B); n = 10 specimens per sample type.

Figure 2.

Log CRP in serum, plasma, capillary DBS, and venous DBS specimens collected at the same time from 52 individuals. Broken line = line of equality. Solid line = linear regression. Unlogged minimum and maximum values across all sample types = 0.03 to 22.4 mg/L CRP.

Figure 3. Bland-Altman plots of differences between log serum CRP and log CRP in plasma (panel A), capillary DBS (panel B), and venous DBS (panel C) plotted against average log CRP concentration; $n = 52$ specimens per sample type. Center line indicates mean difference; upper and lower lines indicate the 95% confidence interval. Broken line indicates the line of equality.

Figure 4.

Effect of long-term storage at -20°C on CRP in serum (panel A) and DBS (panel B); $n = 8$ specimens per sample type per time point. Values are $\text{CRP} \pm \text{SE}$ predicted by linear mixed effects models, with time as a factor. Decline over time was significant for DBS ($p < .0001$) but not for serum ($p = .4$).

Figure 5. Difference between log serum CRP and log CRP in capillary DBS plotted against days of DBS storage at ambient field temperatures in 23 matched sets of specimens collected and stored in a remote field setting. Center solid lines indicates mean difference for specimens stored less than and greater than 21 d at ambient temperatures, broken upper and lower lines indicate the 95% confidence intervals.

Table 1

Summary of specimens used in tests of assay performance and CRP stability.

Specimen Types	n	Collection Site	Volunteers	Use
Serum, plasma, DBS	52 matched sets	lab	US adults	Evaluating CRP assay performance by sample type
DBS	8	lab	US adults	Experiments testing effects of various short-term storage conditions
Serum DBS	8 matched sets	lab	US adults	Assayed at 4 time points to test effects of long-term frozen storage
Serum DBS	23 matched sets	Rural field site	Kenyan adult women	Evaluating effects of field collection and storage on relationship between DBS and serum CRP

Table 2

Pearson correlations for CRP in 52 matched sets of plasma, serum, and DBS (from venous and capillary blood).

	Plasma, mg/L	Capillary DBS, mg/L	Venous DBS, mg/L
Serum, mg/L	.995 *	.979 *	.994 *
Plasma, mg/L		.974 *	.991 *
Capillary DBS, mg/L			.991 *

CRP, c-reactive protein; DBS, dried blood spot

*
p<.001

Table 3

Effect of short-term storage temperature on CRP in DBS. Model estimated means and SEs, mg/L CRP

Baseline	Treatment	Time 1	Time 2	Time 3	Time 4	* p
		7 days	14 days	28 days	42 days	
	-20°C	1.47 ± 0.74	1.52 ± 0.78	1.62 ± 0.78	1.47 ± 0.68	.6
1.81 ± 0.93	21°C	1.27 ± 0.58	1.25 ± 0.54	0.99 ± 0.42	0.87 ± 0.33	.0038
		$\ddagger$ (p=.0906)	$\ddagger$ (p=.0247)	$\ddagger$ (p=.0078)		
	37°C	0.71 ± 0.27	0.46 ± 0.15	0.50 ± 0.21	0.39 ± 0.14	<.0001
		$\ddagger$ (p=.0020)				
		1 cycle	2 cycles	4 cycles	8 cycles	
	-20°C ↔ 21°C	1.64 ± 0.75	1.65 ± 0.74	1.67 ± 0.81	1.50 ± 0.71	.013
					$\ddagger$ (p=.0060)	
2.05 ± 1.03	37°C ↔ 17°C	1.29 ± 0.63	1.19 ± 0.48	1.23 ± 0.52	0.98 ± 0.44	.0023
		$\ddagger$ (p=.0029)				

Linear mixed effects models of log(mg/L CRP) on time for each treatment separately, and time modeled as linear. Random effects were estimated to account for across subject variability in the baseline CRP and for the slope of CRP change per time point. n = 8 specimens for -20°C, 21°C and 37°C; n = 7 specimens for -20°C / 21°C and 37°C / 15°C. Means are estimated from models using unlogged CRP.

* p values for linear change in log(mg/L CRP) across time or cycle.

$\ddagger$ For treatments with a significant change from baseline to time 4, p-values are given to indicate the first time point with results significantly different from baseline in linear mixed effects models with time or cycle as categorical.