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Research Article

Protein C Activity in Dogs: Adaptation of a Commercial Human Colorimetric Assay and Evaluation of Effects of Storage Time and Temperature

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Objectives of this study were to adapt a commercial human protein C (PC) colorimetric assay for use in dogs and to investigate effects of various storage conditions. The human assay was modified by using pooled canine plasma for calibration and by increasing the activation time. PC activity was measured in fresh canine plasma and in plasma stored under various conditions. PC activity of some stored samples was significantly different from that of fresh plasma; however, differences were small. No difference was detected in samples stored under similar conditions but analyzed in different laboratories using similar methodology. Results of this study indicate that the human colorimetric assay is suitable for canine samples if pooled canine plasma is used for calibration, that Clinical and Laboratory Standards Institute sample storage guidelines developed for testing in humans are appropriate for dogs, and that comparisons of results from laboratories using similar methodology are legitimate.

1. Introduction

Protein C is a vitamin K-dependent glycoprotein that is primarily synthesized in the liver [1]. It circulates in plasma as a zymogen of a serine protease and is activated by thrombin-thrombomodulin complex [2]. Activated protein C exerts antithrombotic effects by inactivating coagulation factors Va and VIIIa; additionally, it is reported to have profibrinolytic, anti-inflammatory, and antiapoptotic properties [2]. Many conditions are recognized to cause decreased protein C activity in people, including liver disease, sepsis, and inflammation [2, 3]. In dogs, protein C activity is decreased with a variety of hepatobiliary diseases, especially hepatic failure and congenital portosystemic shunts, and has been used to differentiate congenital portosystemic shunts from microvascular dysplasia secondary to portal hypoplasia [4–6]. Protein C activity is also decreased in dogs with sepsis, congestive heart failure, and naturally acquired food-borne aflatoxicosis [7–10].

A canine-specific protein C assay is not commercially available; therefore, protein C activity in dogs is measured with human assays that employ colorimetric or clotting principles. Several reports cite the use of pooled canine plasma for assay calibration, instead of a commercial human standard; these studies have been performed using various combinations of instruments and assay reagents, including clotting-based and colorimetric (chromogenic substrate-based) assays [4, 6, 8, 9, 11]. Other reported variations in methodology include different activation times, anticoagulant concentrations, and centrifugation settings for separating plasma [8, 11, 12]. However, detailed descriptions of test methodology for measuring protein C concentration in dogs are scarce. The most detailed explanation of modification of a human assay for dogs [11] describes a clotting-based assay [11]. However, most studies of protein C activity in dogs report the use of a colorimetric assay. In adapting a commercially available human colorimetric protein C assay (STA-Stachrom protein C colorimetric assay, Diagnostica

Stago, Asnieres, France) for use in dogs by using canine plasma for calibration, an apparently critical step is the dilution of the plasma to be used as the 100% standard—yet this information is not, to our knowledge, anywhere in the veterinary literature. An accurate, complete description of how this assay can be adapted for use in dogs would be of benefit to any veterinary laboratory interested in adding a protein C assay to its list of test offerings.

Since few veterinary laboratories currently offer protein C assays, most veterinarians must ship samples in order to obtain results. According to Clinical and Laboratory Standards Institute (CLSI) guidelines [13] developed for laboratory testing in people, samples for measurement of human protein C activity are stable (variation $< \pm 10\%$) for 24 months when frozen at -74°C or -24°C [13]. However, relatively little has been published on the stability of canine protein C under different time and temperature conditions. One study [11] found samples to be stable for at least 1 year at -80°C, but the sample size was small ($n = 3$) and no detailed data was reported [11]. Another study [14] found no significant difference in results obtained using plasma frozen at -41°C for 12 to 24 days and those obtained using plasma stored under similar conditions but that underwent an additional freeze-thaw cycle; that study did not include a fresh (unfrozen) plasma control group [14].

The objectives of this study were to adapt a commercial human protein C colorimetric assay for use in dogs, and then to determine the effects of various storage conditions on results. Our hypothesis was that different storage conditions would significantly affect results. Secondary objectives were to compare results from our laboratory with those from a laboratory in another state, and to establish preliminary canine protein C reference values for our laboratory.

2. Materials and Methods

2.1. Animals. The project involved the use of 28 clinically normal adult dogs owned by faculty, staff, or students. All live animal work was approved in advance by an institutional animal care and use committee (University of Tennessee, protocol #1498). Pooled plasma was made using samples from 15 dogs (median age = 7 years; 9 mixed breed, 6 different pure breeds; 9 spayed females, 6 neutered males). Experimental phases 1 and 2 were done using samples from 13 dogs of varying age (phase 1, range = 3–9 years, median = 8 years; phase 2, range = 3–9 years, median = 6.5 years), breed, and sex; samples from 2 dogs were included in both phases.

2.2. Protein C Assay. Samples were analyzed using an automated instrument (STA Compact), and commercially available reagents (STA-Stachrom protein C colorimetric assay, STA-Owren-Koller buffer, STA-System Controls) from the same manufacturer (Diagnostica Stago, Asnieres, France). The assay involves a 2-step reaction in which one reagent (purified *Agkistrodon c. contortrix* venom) activates protein C, another reagent is a chromogenic substrate for the activated enzyme, and the intensity of the resulting color is measured spectrophotometrically as optical density (OD).

Standard curves were established using either a commercial human standard (STA-Unicalibrator, Diagnostica Stago, Asniers, France) according to manufacturer's instructions, or pooled canine plasma as described below. In the regular assay procedure, the 110% human standard calibrator is diluted 1/3, and all test samples are automatically diluted 1/3 (personal communication, Diagnostica Stago technical support); another veterinary laboratory using the same assay for canine samples uses a 1/3 dilution of pooled canine plasma as the 100% standard, and an activation time of 10 minutes instead of the default 5 minutes (personal communication, Cornell Animal Health Diagnostic Center). Based on this information, and on our own experience using the human standard calibrator, we established a canine-specific standard curve using the following dilutions of pooled canine plasma, and a 10-minute activation time (dilution = protein C activity): 1/3 = 100%, 1/4 = 75%, 1/6 = 50%, 1/10 = 30%, 1/20 = 15%, and pure buffer = 0%. To assess precision, two separate curves were generated in immediate succession on the same day, both run in duplicate. Using the second of these curves, a plasma sample from one normal dog was chosen at random and 6 aliquots were measured as part of a single run to determine intra-assay precision. A new canine-specific curve generated one week later (Curve 2) was used for all subsequent assays.

2.3. Samples Collection and Handling

2.3.1. Pooled Plasma. Blood samples (6 mL) were collected by jugular venipuncture from 15 dogs into 3.2% sodium citrate anticoagulant (BD Vacutainer, Becton, Dickenson, and Co., Franklin Lakes NJ). Samples were centrifuged twice for 120 s at 13,700 g (StatSpin, StatSpin, Inc., Norwood MA), then plasma was harvested within 30 minutes of collection, placed in 2 mL screw-cap tubes (Sarstedt, Newton NC), frozen at -80°C within 60 minutes of harvesting, and kept frozen for 12 to 28 d. Samples were thawed by letting the tubes stand at room temperature, and occasionally rolling them between open palms. Pooled plasma was created from 250 μL aliquots of plasma free of gross lipemia or hemolysis; any remaining plasma was refrozen at -80°C.

Phase 1. Blood samples were collected from 9 dogs, and plasma was harvested as described for making pooled plasma. Each plasma sample was analyzed within 60 minutes and the remainder was immediately frozen at -80°C. After 4 days ($n = 7$) or 7 days ($n = 2$), the samples were thawed and divided into 5 aliquots (0.5 mL per aliquot). One of the aliquots was analyzed immediately, and the others were immediately refrozen under different conditions for later testing (Table 1(a)).

Phase 2. Blood samples were collected from 6 dogs, and plasma was harvested as described above. Each plasma sample was divided into 5 aliquots (0.5 mL per aliquot). One of the aliquots was analyzed immediately, and the others were immediately frozen under different conditions for later testing (Table 1(b)).

Table 1: Plasma storage conditions for Phase 1 (a) and Phase 2 (b).

(a)

Sample	Storage conditions
Control	NA (fresh plasma)
Aliquot 1.1	4 d (7 dogs) or 7 d (2 dogs) at −80°C
Aliquot 1.2	4 d (7 dogs) or 7 d (2 dogs) at −80°C, then thawed, refrozen at −20°C, and shipped with a cold pack the next day overnight to an independent laboratory[A]; assay performed 2 d after initial thaw from −80°C
Aliquot 1.3	4 d (7 dogs) or 7 d (2 dogs) at −80°C, then thawed, refrozen at −20°C, and packaged the next day identically to Aliquot 1.2 (package kept at room temperature); assay performed 2 d after initial thaw from −80°C
Aliquot 1.4	4 d (7 dogs) or 7 d (2 dogs) at −80°C, then thawed and refrozen at −80°C for another 4 d
Aliquot 1.5	4 d (7 dogs) or 7 d (2 dogs) at −80°C, then thawed and refrozen at −80°C for another 7 d

[A] Cornell University, Animal Health Diagnostic Center.

(b)

Sample	Storage conditions
Control	NA (fresh plasma)
Aliquot 2.1	7 d at −80°C
Aliquot 2.2	7 d at −20°C
Aliquot 2.3	28 d at −80°C
Aliquot 2.4	28 d at −20°C

2.4. Statistical Analysis. Precision of the protein C assay was determined by calculating coefficients of variation (CV, defined as standard deviation/mean). Results obtained using different types of samples (fresh v. various storage conditions) were compared using commercial software (SAS Version 9.2 of the SAS System for Windows, Cary, NC). Analyses included: Pearson's correlation, Student's t-test for paired comparisons, and repeated measures ANOVA with Shapiro-Wilk test for normality of residuals and Levene's F-test for equal variances. A P-value < .05 was considered significant. For the null hypothesis that there were no differences between control samples and treatments, a Bonferroni correction was employed to adjust for multiple t-tests (for Phase 1 comparisons, $\alpha/n = 0.05/5 = 0.01$; for Phase 2 comparisons, $\alpha/n = 0.05/4 = 0.0125$). For the null hypothesis that there were no differences between treatments, Tukey's post-hoc method was used to adjust for multiple comparisons. Preliminary canine protein C reference values for our laboratory were based on minimum and maximum values from the combined control sample data sets of Phase 1 and Phase 2.

3. Results and Discussion

3.1. Protein C Assay. Based on 4 data points per dilution (2 standard curves, both run in duplicate), the CVs of OD measurements were < 5% (low CV = 2.0%, high CV = 4.8%) for each dilution except pure buffer; for pure buffer, 3 readings were 0.000 and 1 was 0.001. Based on 6 separate measurements of the same sample as part of a single assay run, the CV was 1.4%. Standard curves generated 1 week apart were very similar (Table 2); the instrument output for the second of these curves is shown in Figure 1. Protein C activity values were consistently much lower using the commercial human standard instead of pooled canine plasma to calibrate the assay (data not shown).

Table 2: Comparison of standard curves generated 1 week apart, using pooled canine plasma (Curves 1 and 2), and a curve generated using the manufacturer's standard protocol (Mfr Std). Results for protein C (PC) activity shown here are hypothetical, calculated by inserting arbitrary optical density (OD) values into the regression equations for the curves (Curve 1: c = [1278.276 × d] − 12.397; Curve 2: c = [1257.324 × d] − 9.60; Mfr Std curve: c = [515.734 × d] − 0.608; where c = PC activity and d = OD). Actual instrument output for Curve 2 is shown in Figure 1.

	Protein C activity (%)		
OD	Curve 1	Curve 2	Mfr Std curve
0.02	13	16	10
0.04	39	41	20
0.06	64	66	30
0.08	90	91	41
0.10	115	116	51
0.12	141	141	61

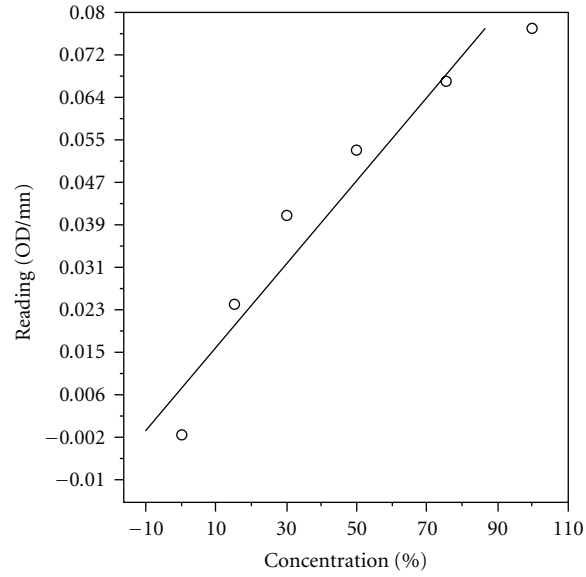

Figure 1: Instrument output for Curve 2. The standard curve was established using the following dilutions of pooled canine plasma (dilution = protein C activity): 1/3 = 100%, 1/4 = 75%, 1/6 = 50%, 1/10 = 30%, 1/20 = 15%, and pure buffer = 0%.

Phase 1. Phase 1 results are summarized in Table 3(a).

Correlation for comparisons of fresh samples with samples stored under the 5 different conditions ranged from $r = 0.76$ to $r = 0.93$. Correlations for comparisons of one

Table 3: Protein C activity results of fresh (control) and stored plasma for Phase 1 (a) and Phase 2 (b). Cross symbols indicate results of comparisons of control and treatment groups, and alphabetical symbols indicate results of comparisons of different treatment groups; results for groups including the same symbols are not significantly different.

(a) Protein C Activity (%, mean ± SD).

Control	4–7 d, −80°C	4–7 d, −80°C; thawed, 2 d, −20°C, send-out	4–7 d, −80°C; thawed, 2 d, −20°C, in-house	4–7 d, −80°C; thawed, 4 d, −80°C	4–7 d, −80°C; thawed, 7 d, −80°C
$95 \pm 10^{\dagger}$	$100 \pm 15^{\dagger,a,b,c}$	$97 \pm 14^{\dagger,b}$	$102 \pm 11^{\dagger,a,b,c}$	$111 \pm 16^{\ddagger,c}$	$105 \pm 15^{\ddagger,a,b,c}$

(b) Protein C Activity (%, mean ± SD).

Control	7 d, −80°C	7 d, −20°C	28 d, −80°C	28 d, −20°C
$94 \pm 12^{\dagger}$	$94 \pm 11^{\dagger,a}$	$97 \pm 11^{\ddagger,a}$	$88 \pm 10^{\ddagger,b}$	$87 \pm 10^{\ddagger,b}$

storage condition with other storage conditions ranged from $r = 0.77$ to $r = 0.98$.

Samples stored for 4 to 7 d at −80°C, then thawed and refrozen at −80°C for either an additional 4 d or 7 d, had significantly higher protein C activity than control samples. Samples frozen for 4 to 7 d at −80°C, then thawed and refrozen at −20°C for an additional 2 d and measured in-house, had higher protein C activity than control samples; this difference was marginally significant ($P < .02$).

There was no significant difference in results determined by two different laboratories using samples stored under the same conditions (4 to 7 d at −80°C, then thawed and refrozen at −20°C for an additional 2 d). Samples stored for 4 to 7 d at −80°C, then thawed and refrozen at −20°C for an additional 2 d and measured by a laboratory in another state, had significantly lower protein C activity than samples stored for 4 to 7 d at −80°C, then thawed and refrozen at −80°C for an additional 4 d. Samples stored for 4 to 7 d at −80°C had lower protein C activity than samples stored for 4 to 7 d at −80°C, then thawed and refrozen at −80°C for an additional 4 d; this difference was marginally significant ($P = .0503$).

Phase 2. Phase 2 results are summarized in Table 3(b).

Correlation was high ($r > .95$) for all comparisons of fresh samples with samples stored under various conditions, and for all comparisons of one storage condition with other storage conditions.

Samples stored for 7 d at −20°C had significantly higher protein C activity, and samples stored for 28 d at either temperature (−20°C or −80°C) had significantly lower protein C activity, than control samples.

Samples stored for 7 d had significantly higher protein C activity than samples stored for 28 d, irrespective of storage temperature.

3.2. Preliminary Reference Values. Based on the combined control sample data sets of Phase 1 and Phase 2, the minimum and maximum values for protein C activity were 80% and 115%, respectively. We are using these as preliminary lower and upper reference limits, respectively, in our laboratory.

3.3. Discussion. This study was motivated by our desire to measure canine protein C activity in our laboratory, and to learn more about effects of different storage conditions on test results. Limitations of the study included relatively small sample sizes and fragmentation into two experimental phases to investigate storage effects. Despite these limitations, we expect this report to be useful to anyone interested in establishing a canine protein C assay in their own laboratory or seeking more information about effects of different storage conditions (including shipping samples to another institution for analysis).

In our laboratory, the human standard for assay calibration had unacceptably low results (Table 2), similar to what has been reported by others [11]. This phenomenon may occur because the protein C-activating reagent, a highly purified extract of the venom of the *Agkistrodon c. contortrix* snake, does not work as effectively on the canine protein. Investigators evaluating effects of a similar extract using a clotting-based assay found canine samples to be markedly hyporesponsive compared to samples from humans, cattle, and horses [15]; the precise reason for this diminished effect is not clear, but the canine protein C is only partially homologous (72%) with the human form [15]. Nevertheless, our findings and those of others indicate that *Agkistrodon c. contortrix* venom works sufficiently on canine samples to enable the measurement of protein C activity if pooled canine plasma is used to calibrate the assay. Using this approach, our preliminary reference values of 80–115%, based on minimum and maximum values because of the relatively small sample size, are consistent with those reported by Toulza et al. (75–135%) [4] and Bauer et al. (76–119%) [4, 11]. The precision of the assay was acceptable: our coefficients of variation were lower than reported by others [4, 8, 11] and met the performance goals for diagnostic application in humans [4, 8, 11, 16].

We investigated effects of storage conditions different from those previously reported. In one previous study, plasma samples were stored at −20°C for 2 months or −60°C for 10 months before analysis [15]. In another study, samples were stored for a maximum of 3 weeks at −80°C, although the analysis of aliquots of 3 specimens also stored for 13 months at −80°C found no change in results [11]. In a third study, canine plasma was stored up to 2 months at −70°C before batches of samples were shipped to an outside laboratory for analysis [8]. We used conventional statistical methods to test for significant differences in protein C activity. Other investigators [17, 18] have defined stability

of the analyte as a change of $<\pm 10\%$ from the initial value [17, 18]. Using either approach to our data, the protein C activity of some stored samples was different from that of fresh plasma. In addition, some differences were not statistically significant but had low *P*-values, suggesting that the lack of significance could be due to small sample size. However, the mean values for all treatment groups were within the reference limits and the differences, even when statistically significant, were small; moreover, only samples that underwent two freeze-thaw cycles exceeded 10% variation, and then only marginally.

Based on these findings, we interpret the differences in results after the experimental storage conditions to be of little to no clinical significance. This interpretation is consistent with the findings of investigators who have studied the effects of different storage conditions on human or canine protein C activity [11, 14, 17–20]. Clinical and Laboratory Standards Institute guidelines [13] developed for laboratory testing in people indicate that specimens for protein C testing should be kept at room temperature and centrifuged and tested within 4 h from time of collection, or, if testing is not completed within 4 hours, platelet-poor plasma should be frozen at -20°C for up to 2 weeks or at -70°C for long-term storage [13]. Based on our results and those of others, we believe the CLSI sample storage guidelines developed for testing in humans are also appropriate for testing in dogs. The CLSI guidelines do not include any specific recommendations regarding effects of repeated freeze-thaw cycles on protein C activity. Our recommendation is to avoid repeated freeze-thaw cycles, unless future work addressing this issue more definitively indicates otherwise.

Protein C activity was not significantly different in samples stored under similar conditions but analyzed in different laboratories. The two laboratories in this study used the same instruments and assays but different batches of pooled canine plasma to calibrate the assay. The lack of significant difference suggests that data generated from laboratories using essentially the same methodology can be legitimately compared. Variability in protein C results within and between laboratories has been the subject of study in human laboratories but, to our knowledge, not in veterinary laboratories [21].

4. Conclusions

In summary, this report describes how we adapted a commercial human colorimetric protein C assay for use in dogs. Our preliminary reference values are consistent with those of other investigators using colorimetric or clotting-based assays, and the precision of the assay compared favorably with other reports and with performance goals established for human laboratories. In addition, we report the effects of previously unreported storage conditions on test results. We believe that the differences between fresh samples and those stored under the study conditions were of little to no clinical significance, that CLSI guidelines developed for testing in humans are also appropriate for dogs, and that comparisons of results from laboratories using essentially the same methodology are likely to be valid.

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