

# An ex vivo evaluation of efficacy of refrigerated canine plasma

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## Abstract

**Objectives** – To determine thawing times of fresh frozen plasma (FFP), and to evaluate the activity of hemostatic proteins (coagulation factors V, VII, VIII, IX, X, and fibrinogen), clotting times (prothrombin time and activated partial thromboplastin time), and sterility of canine plasma stored refrigerated.

**Design** – Prospective laboratory-based study.

**Setting** – Veterinary teaching hospital blood bank.

**Interventions** – Phase 1: Six units of canine FFP were retrieved from the blood bank and thawed individually in a warm water bath. Time for thaw was recorded in minutes and reported as mean  $\pm$  SD. Phase 2: One unit of fresh whole blood was collected from 9 dogs and processed routinely. Resulting plasma was divided into 2 aliquots, 1 stored as refrigerated plasma (RP) and 1 as frozen plasma. Samples from the RP were taken at 0, 1, 5, 7, and 14 days and from the FFP at days 0 and 14 for determination of clotting factor activity (V, VII, VIII, IX, and X and fibrinogen) and clotting times. Coagulation factors and clotting times were analyzed using a mixed effects linear model for ANOVA, comparing changes over time as well as differences between groups. For all comparisons, a *P* value of  $<0.05$  was considered significant. Batch bacterial aerobic and anaerobic cultures of the RP samples were submitted on days 7 and 14 and from the frozen plasma on day 14.

**Measurements and Main results** – Time to thaw for FFP units was  $34.7 \pm 1.38$  minutes. Refrigerated storage resulted in significant decreases in the activity of all clotting factors and a subsequent prolongation in clotting times. However, no values were outside of the reference interval. All bacterial cultures yielded no growth.

**Conclusions** – Refrigerated storage results in only minor loss of coagulation factor activity in canine plasma. The use of RP, therefore, may be a viable option in high-volume veterinary hospitals for rapid correction of coagulopathy in critical care patients.

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**Keywords:** clotting factor activity, hemostasis, transfusion

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## Abbreviations

AABB	American Association of Blood Banking
aPTT	activated partial thromboplastin time
FFP	fresh frozen plasma
FP	frozen plasma
FWB	fresh whole blood
pRBC	packed red blood cells
PT	prothrombin time
RP	refrigerated plasma
TCSVM	Tufts Cummings School of Veterinary Medicine

## Introduction

Blood transfusions are an important component of treating injured and ill dogs in emergency and critical care

medicine. Over the last 30 years, transfusion medicine in dogs has evolved from using solely fresh whole blood (FWB) collected as needed for in-house recipients, to the development of local and national veterinary blood banks, which focus on providing the safe and prompt delivery of component therapy, including fresh frozen plasma (FFP) and packed red blood cells (pRBC) for transfusion.<sup>1</sup> FFP and pRBC are prepared by centrifugation of a unit of FWB within 8 hours of collection, and separating the plasma and red cell components. PRBCs, stored at 4°C in a commercially available preservative,<sup>a</sup> are considered viable for 35 days, while FFP maintains acceptable clotting factor activity for up to 1 year if stored at -20°C.<sup>1</sup>

Coagulopathy is not uncommon in critically ill or injured dogs, often associated with dysregulated coagulation and fibrinolysis (eg, acute traumatic coagulopathy, disseminated intravascular coagulation), antagonism (eg, anticoagulant rodenticide toxicosis, citrate in pRBCs especially associated with massive transfusion), or dilution (eg, with IV infusion of large volumes of isotonic crystalloids or colloids). While anemia may be treated immediately with pRBC, rapid correction of coagulopathy requires transfusion with plasma or FWB. FWB requires collection from a donor dog, which may or may not be readily available, while FFP needs to be thawed prior to transfusion. This time delay from initiation of thawing to transfusion represents a potential risk to a dog with massive hemorrhage or active bleeding from a coagulopathy.

Storing plasma frozen is considered the best method to preserve clotting factors, including the labile factors V and VIII.<sup>2</sup> The current recommendations in veterinary medicine are to maintain plasma frozen and thaw only as needed.<sup>1</sup> However, thawing one unit of plasma (~250 mL) may be time consuming, and in critical patients, major blood loss could occur during the time required to thaw a unit of FFP. The actual time required to thaw FFP has not been reported in a clinical setting.

In high volume human trauma centers, plasma is stored both as refrigerated (liquid) and frozen plasma (FP).<sup>3</sup> Refrigerated plasma (RP) is available immediately for transfusion, similar to pRBC. While stocking RP would allow immediate transfusion in unstable dogs, the utility of RP is dependent upon the maintenance of adequate coagulation factor activity, as urgent transfusion would be required almost universally to support clinically relevant coagulopathy.

Previous human studies have demonstrated that coagulation factors at adequate levels for hemostasis can be recovered from human plasma samples stored at 4°C for periods extending to 28 and 35 days.<sup>4,5</sup> Massive transfusion protocols commonly include the use of RP, with recommendation in people for transfusion of a 1:1 ratio

of pRBC to plasma, alone or in combination with cryoprecipitate or platelet concentrates.<sup>6</sup>

Prior studies have documented that storage of canine plasma at 4°C results in a statistically significant decrease in activity of coagulation factors VIII, IX, and XI within 48–72 hours; however, factor activity remained within the normal reference interval.<sup>7</sup> As such, the influence on clinical efficacy is likely minimal. This is supported by other studies showing that refrigeration of canine plasma for up to 48 hours does not result in significant increases of prothrombin time (PT) or activated partial thromboplastin time (aPTT).<sup>8,9</sup> While these studies suggest that RP is likely to be efficacious if used within 48–72 hours, no studies have evaluated the stability of canine RP after longer periods of storage.

In addition to preserving clotting factors, plasma has been stored frozen to prevent bacterial overgrowth of units from infected donors or from contamination during collection. While the rate of bacterial contamination of liquid blood products is unknown, owing to variable methods of prevention and bacterial detection, rates of contamination as high as 13% for refrigerated whole blood have been reported in human medicine.<sup>10</sup> Thus, while RP is an appealing addition to the transfusion armamentarium of the veterinarian, further investigation is required prior to the routine recommendation of its use in clinical practice.

The goals of this study were (1) to determine length of time for one unit of conventionally stored FFP to thaw using a water bath, (2) to evaluate the coagulation stability of canine plasma during a 2-week period of refrigeration by measuring the activity of coagulation factors and clotting times, and (3) to evaluate units of RP for the growth of bacteria.

## Materials and Methods

### Plasma thaw time analysis

Six units of FFP were selected from the Tufts University Cummings School of Veterinary Medicine (TCSVM) blood bank, removed from frozen storage, and immediately, individually thawed in a 37°C warm water bath. Time was recorded as the time from full submersion of each unit until fully thawed, in minutes. The units were not agitated during this time and each was considered to be thawed when ice particles could no longer be detected by visual inspection or palpation of the unit. The warm water bath was allowed to reequilibrate to 37°C between thawing each unit.

### Plasma collection

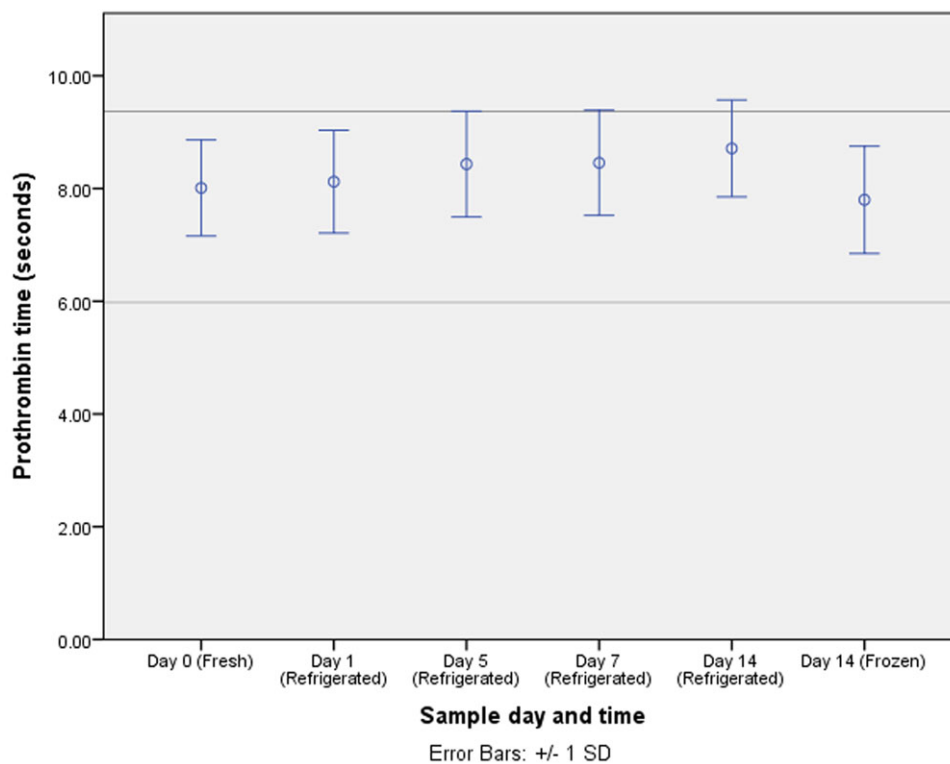
Whole blood (450 mL) was drawn from 9 healthy dogs belonging to students or staff at the TCSVM. After

**Table 1:** Comparison of clotting factor activity and performance of functional clotting assays when using refrigerated plasma and frozen plasma. aPTT, activated partial thromboplastin time; D0, day 0; D1, day 1; D5, day 5; D7, day 7; D14, day 14; PT, prothrombin time; RP, refrigerated plasma; FP, frozen plasma; FV, factor V; FVII, factor VII; FVIII, factor VIII; FIX, factor IX; FX, factor X.

Coagulation value (reference interval)	RP					FP		RP versus FP D14 P
	D0	D1	D5	D7	D14	D0	D14	
PT (5.9–9.3 seconds)	8.0 ± 0.9	8.1 ± 0.9	8.4 ± 0.9	8.5 ± 0.9	8.5 ± 0.9*	8.0 ± 0.8	7.8 ± 1.0*	<0.001
aPTT (9.9–20.4 seconds)	16.9 ± 2.9	17.8 ± 3.2	17.8 ± 2.9	17.9 ± 3.0	18.5 ± 3.2*	16.9 ± 2.9	16.9 ± 2.7	<0.001
Fibrinogen (73.4–410 mg/dL)	193 ± 41	180 ± 42	177 ± 41	178 ± 42	170 ± 39*	193 ± 41	193 ± 48	<0.001
FV (>50% activity)	125 ± 37	129 ± 52	116 ± 35	114 ± 33	114 ± 44	125 ± 37	125 ± 31	0.42
FVII (>50% activity)	139 ± 62	122 ± 66**	125 ± 58	119 ± 64	120 ± 63	139 ± 62	118 ± 387	0.75
FVIII (>50% activity)	122 ± 99	106 ± 115	110 ± 122	104 ± 108	98 ± 123	122 ± 99	112 ± 103	0.14
FIX (>50% activity)	123 ± 133	137 ± 244	142 ± 272	147 ± 282	148 ± 330	123 ± 133	131 ± 197	0.65
FX (>50% activity)	79.9 ± 7.8	76.3 ± 4.7	73.6 ± 6.8	72.7 ± 7.6	73.3 ± 7.3*	79.9 ± 7.8	73.6 ± 7.0*	0.93

\*P < 0.05 comparing D0 with D14.

\*\*P < 0.05 when comparing with previous sampling time point.

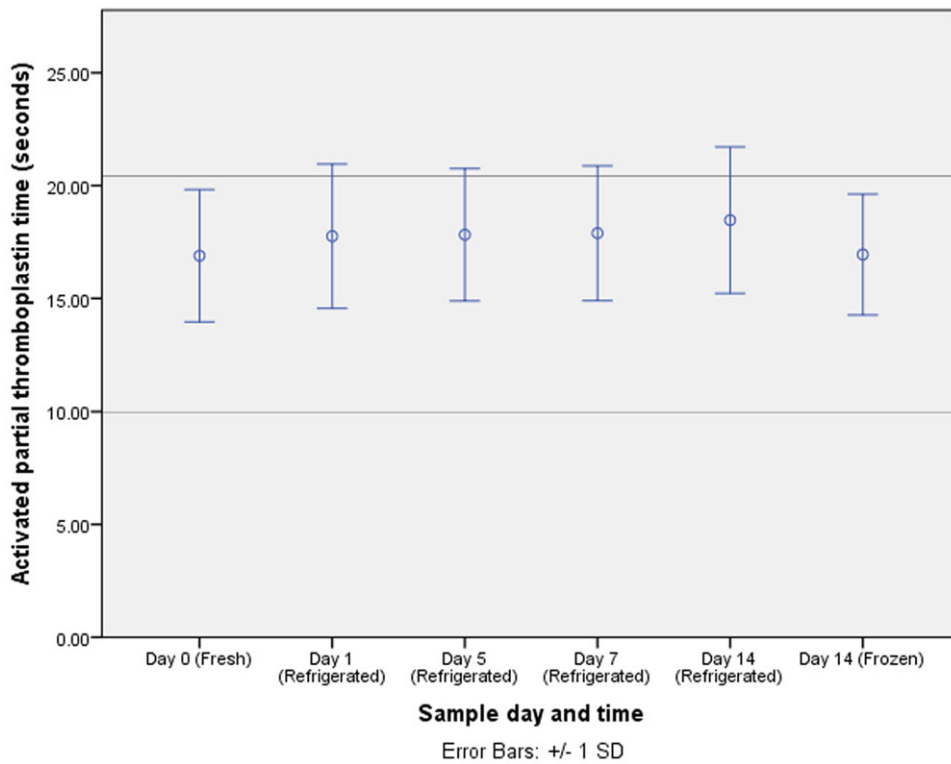


**Figure 1:** Graphic representation of changes in prothrombin time in averaged refrigerated plasma and frozen plasma samples over a 14 day period of time. Refrigerated plasma has been sampled multiple times throughout this process.

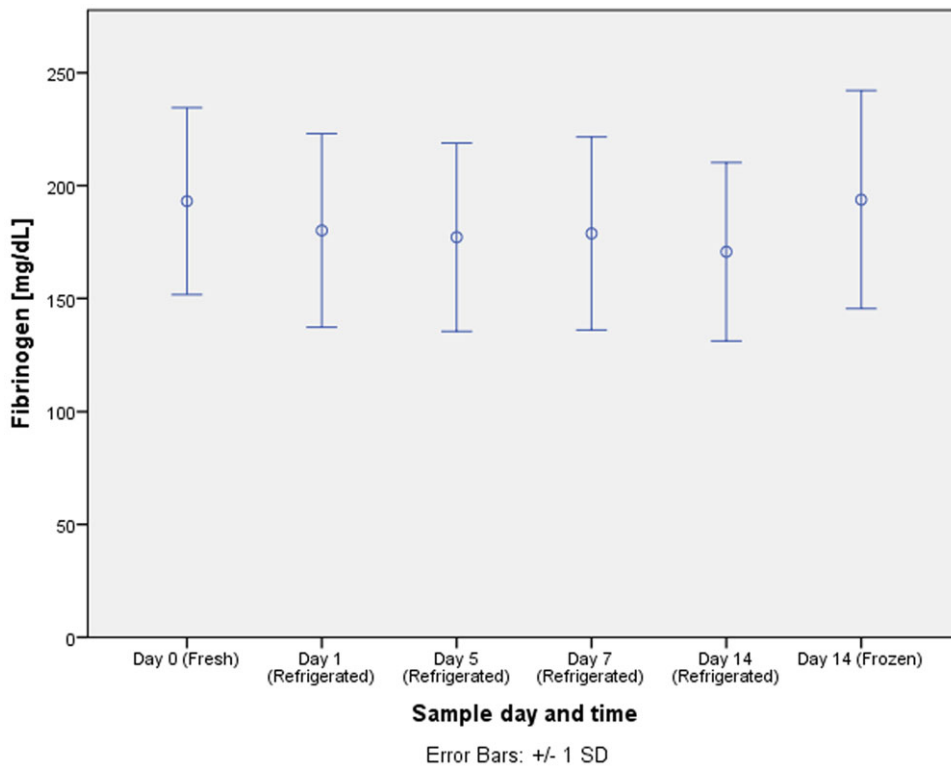
collection, the blood was separated into pRBC and plasma via centrifugation at 5,000 × g for 15 minutes at 4°C. The units of pRBC were placed in the blood bank for routine use and not evaluated further. The plasma units were then each aseptically separated into 2 sub-units within 2 hours of collection, with 9 subunits frozen at –20°C (FP) and the other 9 subunits stored in a medical grade blood refrigerator at 4°C (RP). The time of initial storage was designated as “Day 0.” Aliquots from each plasma unit were collected prior to storage and labeled

“Day 0” and stored at –80°C. The study was approved by the Clinical Sciences Review Committee and all owners provided informed consent for their dog’s enrollment in this study.

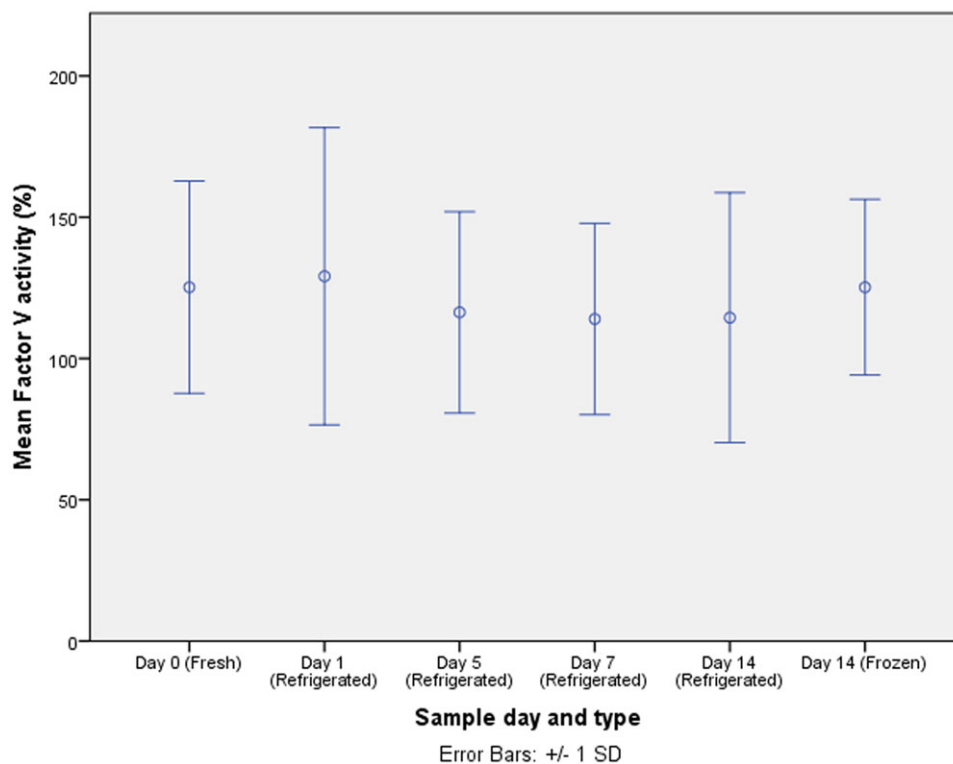
The RP units were stored with a sampling site coupler<sup>b</sup> in place and 4 mL aliquots were aseptically sampled from each unit after 24 hours of storage (Day 1) and on days 5, 7, and 14. Each aliquot was subsequently stored at –80°C until batch analysis. On day 14, the FP subunits were thawed in a warm water bath at 37°C, and 4 mL



**Figure 2:** Graphic representation of changes in activated partial thromboplastin time in averaged refrigerated plasma and frozen plasma samples over a 14 day period of time.



**Figure 3:** Graphic representation of changes in fibrinogen in averaged refrigerated plasma and frozen plasma samples over a 14 day period of time. Refrigerated plasma has been sampled multiple times throughout this process.



**Figure 4:** Graphic representation of changes in coagulation factor V activity in averaged refrigerated plasma and frozen plasma samples over a 14 day period of time. Refrigerated plasma has been sampled multiple times throughout this process. Intrassay coefficient of variation is 3.3%.

aliquots were aseptically obtained from each subunit and stored at  $-80^{\circ}\text{C}$ . Plasma remaining after sampling was discarded and not used for transfusion.

### Measurement of clotting times

PT, aPTT, and fibrinogen were evaluated in the TCSVM Coagulation laboratory using quantitative methodology.<sup>c</sup>

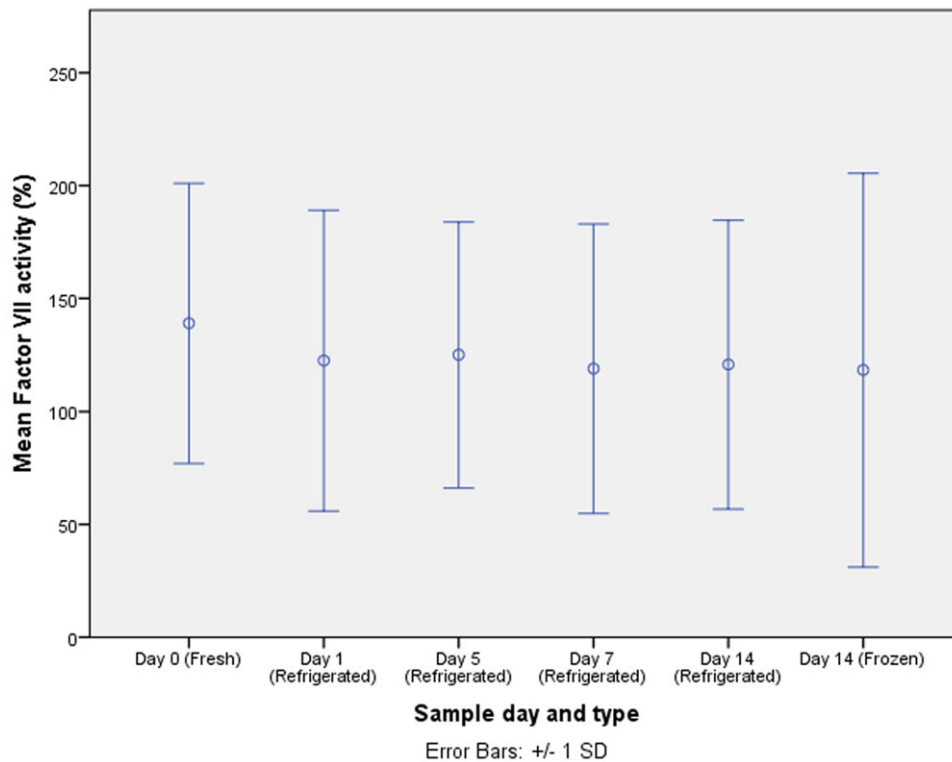
### Coagulation factor analysis

Analyses of factors V, VII, VIII, IX, and X were performed at the Comparative Coagulation Section at Cornell University's Animal Health Diagnostic Center. The plasma samples were stored at  $-80^{\circ}\text{C}$  until thawed in a water bath at  $37^{\circ}\text{C}$  immediately before assay. All of the factor assays were performed on the same day, using a single lot of commercial aPTT and PT reagents, substrate deficient plasmas, and canine standard plasma. The canine standard plasma was prepared at the Coagulation Laboratory as pooled plasma from 20 healthy dogs and stored in single-use aliquots at  $-80^{\circ}\text{C}$ . The standard plasma had an assigned factor activity of 100%. Intrinsic factor coagulant activity assays (factors VIII:C and IX:C) were performed using a modified one-stage aPTT technique

with a commercial aPTT reagent<sup>d</sup> and canine congenital deficient factor VIII and factor IX substrate plasmas, as previously described.<sup>11</sup> The coagulant activities of factors V, VII, and X were performed using a modified one-stage PT technique, a rabbit thromboplastin reagent,<sup>e</sup> human or canine substrate deficient plasmas (for factor V:C and VII:C, respectively), and an adsorbed, artificially depleted bovine plasma and a snake-venom activator (for factor X:C).<sup>f12,13</sup> The clotting times for the test plasmas were determined and reported, after log-log transformation, as percentage activity compared with dilutions of the canine plasma standard. The intra-assay coefficient of variation for each factor assay was determined based on analyses of a separate dilution of the standard plasma assayed before, during, and after the test run of the submitted samples. The percent coefficient of variation was calculated as the SD divided by the mean of the 3 determinations.

### Aerobic and anaerobic bacterial cultures

Aliquots from each RP unit (1 mL each) were inoculated into a single blood culture vial<sup>g</sup> on days 7 and 14. A batch culture from the FP units was performed on day 14. Bacterial cultures were performed at a national reference laboratory.<sup>h</sup>



**Figure 5:** Graphic representation of changes in coagulation factor VII activity in averaged refrigerated plasma and frozen plasma samples over a 14 day period of time. Refrigerated plasma has been sampled multiple times throughout this process. Intrassay coefficient of variation is 6.5%.

### Statistical analysis

Time for FFP units to thaw is reported as mean  $\pm$  SD. Coagulation factor activity, clotting times, and fibrinogen concentrations were analyzed across time and among groups using a mixed effects linear model of ANOVA, comparing the separate values for each daily sample to the immediately preceding value as well as a comparison to baseline. For all comparisons, a  $P$  value of  $< 0.05$  was considered significant. Aerobic and anaerobic bacterial cultures were reported as positive or negative for growth.

## Results

### Plasma thaw time

All FFP units required more than 30 minutes to be fully thawed ( $34.7 \pm 1.38$  minutes).

### Prothrombin time

In RP, the PT lengthened significantly between days 0 and 14 ( $P < 0.001$ ), and daily between days 1 and 5 ( $P = 0.002$ ) and days 7 and 14 ( $P = 0.012$ ). In FP, the PT shortened significantly between days 0 and 14 ( $P = 0.03$ ). Between RP and FP, there was a significant differ-

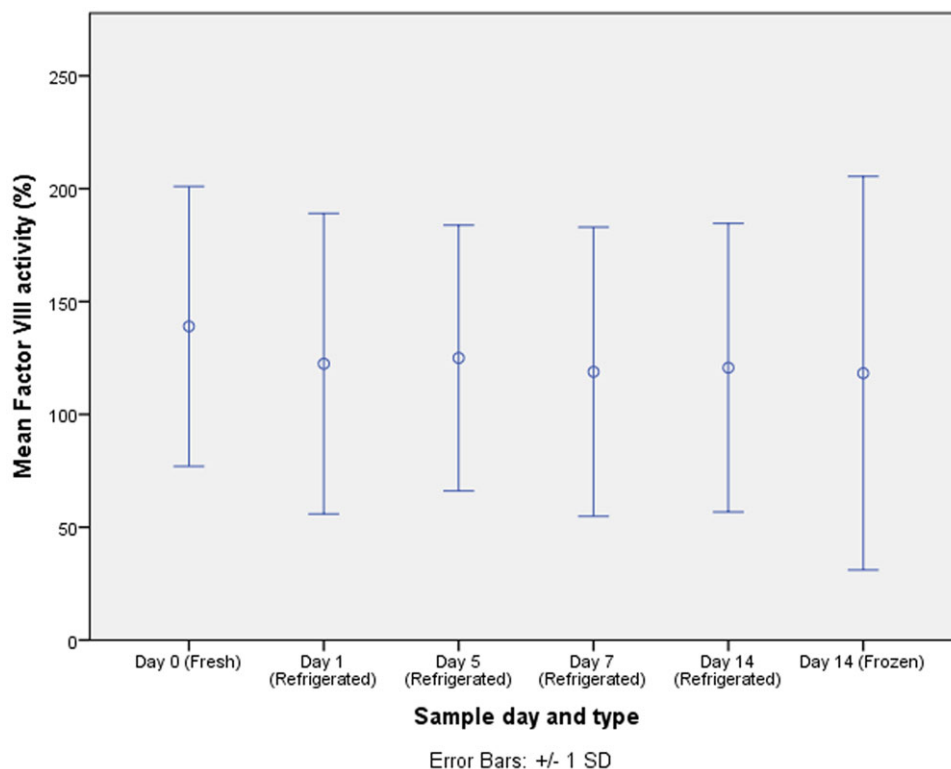
ence at day 14 ( $P < 0.001$ ). However, all PT measurements remained within the reference interval (Table 1, Figure 1).

### Activated partial thromboplastin time

In RP, the aPTT lengthened significantly between days 0 and 14 ( $P < 0.001$ ), and daily between days 0 and 1 ( $P = 0.001$ ) and between days 7 and 14 ( $P = 0.02$ ). In FP, there was no change ( $P = 0.82$ ) between days 0 and 14. Between RP and FP, there was a significant difference at day 14 ( $P < 0.001$ ). All aPTT results remained within the reference interval (Table 1, Figure 2).

### Fibrinogen

In RP, the fibrinogen concentration decreased significantly between days 0 and 14 ( $P < 0.001$ ), and daily between day 0 and 1 ( $P = 0.005$ ) and between days 7 and 14 ( $P = 0.07$ ). In FP, there was no change ( $P = 0.88$ ) between days 0 and 14. While in the full dataset there was not a significant decrease in the fibrinogen concentration in RP between days 5 and 14 ( $P = 0.15$ ), when we excluded an influential point the decrease became statistically significant ( $P = 0.036$ ). Between RP and FP, there



**Figure 6:** Graphic representation of changes in coagulation factor activity VIII in averaged refrigerated plasma and frozen plasma samples over a 14 day period of time. Refrigerated plasma has been sampled multiple times throughout this process. Intrassay coefficient of variation is 9.7%.

was a significant difference at day 14 ( $P < 0.001$ ). All fibrinogen concentrations remained within the reference range (Table 1, Figure 3).

#### Clotting factors

##### **Factor V:**

In RP and FP, there was no significant change in factor V activity over time during storage and all values were within the reference interval (Table 1, Figure 4).

##### **Factor VII:**

In RP, factor VII activity decreased between days 0 and 14 ( $P = 0.03$ ) and day 0 to 1 ( $P = 0.04$ ). There was no further significant decrease after day 1. In FP, there was a decrease in factor VII activity between days 0 and 14 ( $P = 0.01$ ). There was no difference between groups at day 14. All factor VII concentrations remained within the reference interval (Table 1, Figure 5).

##### **Factor VIII:**

In RP, factor VIII activity decreased between days 0 and 14 ( $P = 0.01$ ). In FP, there was no significant decrease

in factor VIII activity between days 0 and 14 ( $P = 0.13$ ). There was no difference between groups at day 14. All factor VIII concentrations remained within the reference range (Table 1, Figure 6).

##### **Factor IX:**

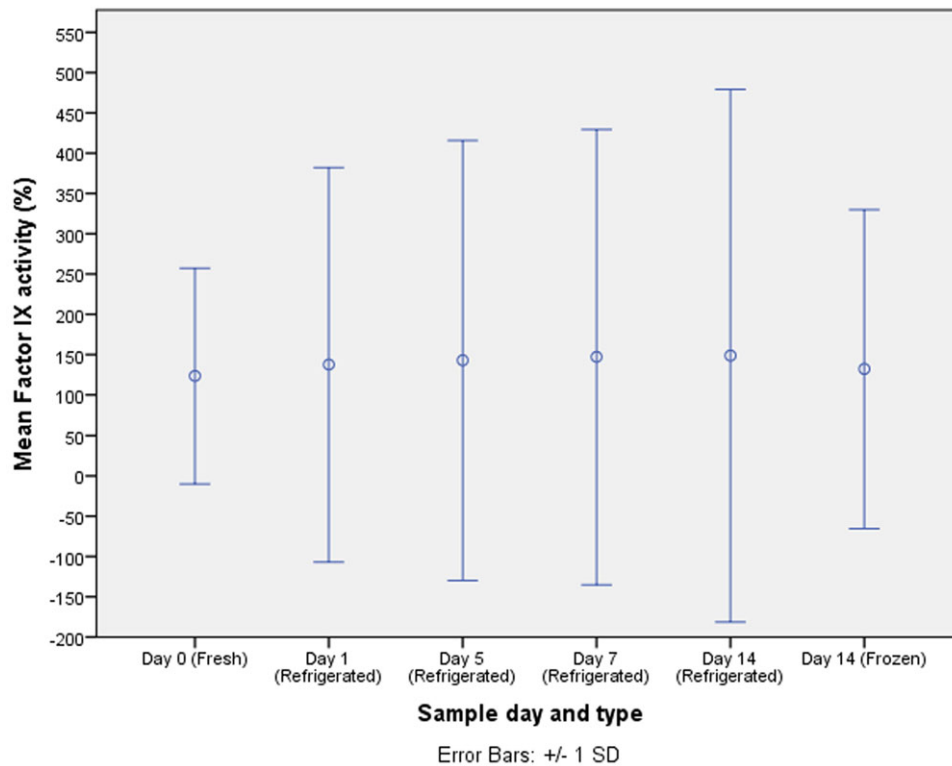
No significant changes occurred in Factor IX activity in RP or FP during storage. Additionally, there was no difference between groups at day 14 (Table 1, Figure 7).

##### **Factor X:**

In RP, the factor X concentration decreased significantly from day 0 to day 14 ( $P = 0.02$ ); however, there were no significant differences in day-to-day measurements. There was no difference from day 0 to day 14 in FP, although there was a difference ( $P = 0.03$ ) between RP and FP at day 14. All factor X concentrations remained in the reference range (Table 1, Figure 8).

#### **Bacterial culture results**

Aerobic and anaerobic bacterial cultures obtained on day 7 and day 14 for RP and on day 14 from FP yielded no growth.



**Figure 7:** Graphic representation of changes in coagulation factor activity IX in averaged refrigerated plasma and frozen plasma samples over a 14 day period of time. Refrigerated plasma has been sampled multiple times throughout this process. Intrassay coefficient of variation is 2.0%.

## Discussion

The results of this study support that plasma may be stored for up to 14 days in the refrigerator without clinically relevant loss of factor activity and subsequent clotting function, or evidence of bacterial contamination. There were significant increases in coagulation times as well as significant degradation of fibrinogen over the course of the 14 day period in the refrigerated samples. This significant difference was noted within the first 24 hours for both aPTT and fibrinogen and within the first 5 days of storage for PT. In addition, significant differences were noted between the frozen and RP samples on day 14. However, in no sample was the fibrinogen, PT or aPTT outside the reference range, supporting the presumptive lack of clinical relevance.

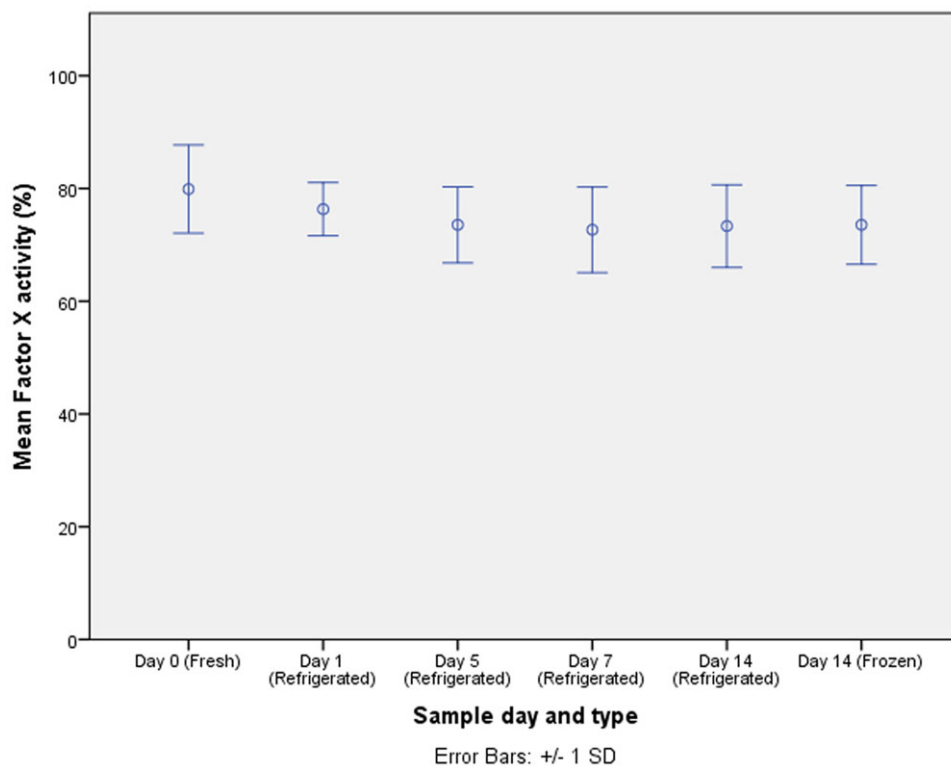
Prior studies with canine RP have not found evidence of clinically significant prolongation of PT or aPTT when stored for up to 24 hours,<sup>9</sup> 4 days,<sup>7</sup> and 7 days.<sup>8</sup> These results are consistent with our study, now documenting that PT and aPTT are not prolonged to a clinically significant extent (ie, they remain within reference intervals) with refrigerated storage of canine plasma out to 14 days.

Previous studies have also evaluated the stability of fibrinogen in canine plasma with refrigerated storage. One study documented a significant decrease over 24

hours of refrigerate storage;<sup>9</sup> however, baseline values were extremely high in that study. Other studies have shown <10% decrease in fibrinogen concentrations in canine RP when stored for 4<sup>7</sup> and 7 days.<sup>8</sup> Our study documented an approximately 20% decrease in fibrinogen concentrations over 14 days; however, since the fibrinogen concentration remained within the reference interval, this change is not thought to be of biological significance. Only one study has previously evaluated changes in clotting factor activity over time in canine plasma during refrigerated storage. Over 4 days of refrigerated storage, there was no statistically significant change in the activity of factors II, V, VII, X, or XII, when compared to baseline. Similarly, our study did not find that the activity of factors V, VII, or IX changed over 14 days of refrigerated storage. We did not analyze factors II or XII. Even those clotting factors that did demonstrate a statistically significant decline in activity over the 14 days of refrigerated storage (ie, factors VIII and X) had activities that remained within the reference interval at day 14, suggesting minimal clinical significance.

According to the American Association of Blood Banking (AABB) standards, RP can be stored for 5 days. They advise against its use in patients with deficiencies in factors V and VIII due to rapid reduction in factor activity at refrigerated temperatures.<sup>3</sup> However, over





**Figure 8:** Graphic representation of changes in coagulation factor activity X in averaged refrigerated plasma and frozen plasma samples over a 14 day period of time. Refrigerated plasma has been sampled multiple times throughout this process. Intrassay coefficient of variation is 4.0%.

5 days time in refrigerated storage, FV activity in canine plasma in our study decreased by only 9% and FVIII decreased 12% suggesting that some clinical benefit may remain throughout this period. Additionally, in the current study, no significant differences for factors V and VIII were noted between the RP and FP when compared at day 14. This is consistent with recent human data, which have reported maintenance of therapeutic levels of factors V and VIII in plasma units stored at 6°C for 10 days.<sup>2</sup>

In addition to storage recommendations based on efficacy the AABB recommendations take into account the relative risk of bacterial contamination over time. Traditionally, thawed human plasma is recommended to be discarded within 5 days to limit the potential of bacterial contamination.<sup>3</sup> In the current study, RP units showed no evidence of bacterial growth when batch cultured at day 7 and day 14. However, it has been reported that bacteria found in refrigerated blood products are psychrophiles, capable of rapid growth at refrigerator temperatures (1–4°C) for up to 42 days. This study did not account for prolonged incubation at a refrigerated temperature as standard aerobic and anaerobic bacterial cultures were performed at a national reference laboratory.<sup>e</sup> Although the batch culture technique was adopted due to financial

constraints of the study, the authors have no reason to believe, based on the existing literature, that this would significantly reduce our ability to identify bacterial growth via incubated culture. More recently, human blood banks have adopted the protocol of screening platelet concentrate units (stored at room temperature) via PCR for bacterial DNA.<sup>10</sup> Though this may prove useful to identify bacterial DNA, it cannot distinguish between DNA fragments and viable bacteria, and it is not routinely used for screening of refrigerated blood products at this time in human medicine. Given the financial limitations of the study, the limitations of PCR, and the fact that liquid plasma screening by PCR is not routinely performed in human medicine, this was not pursued in our study.

Concomitant with the growth of blood banking, there has been an introduction of synthetic colloids, which has nearly abolished the need for plasma transfusion for colloidal support. Plasma for therapy of pancreatitis has also decreased due to lack of clinical efficacy.<sup>14</sup> Thus, there exists a relative abundance of FFP in most critical care settings with in-house blood banks, which should effectively diminish concerns surrounding potential waste if a RP program is started. In severe trauma or illness, massive or large volume transfusion may be required

and thawing time of FFP represents a severe obstacle. This study has shown that for a high-volume emergency clinic, storing several units of plasma in a refrigerated state may bypass this temporal obstacle.

Although this ex vivo study did not investigate clinical efficacy of the stored RP units, the maintenance of normal coagulation times and fibrinogen concentrations supports that RP would be expected to be effective in correcting coagulopathy. The advantages of readily available source of clotting factors may negate any potential disadvantage of potential plasma loss. Further evaluation of the clinical use of RP is warranted in acute coagulopathy in dogs.

### Acknowledgments

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### Footnotes

- <sup>a</sup> Adsol, Fenwal Inc, Lake Zurich, IL.
- <sup>b</sup> Sampling Site Coupler, Fenwal Inc.
- <sup>c</sup> IL ACL Elite coagulation analyzer, Diamond Diagnostics, Holliston, MA.
- <sup>d</sup> Dade Actin, Siemens Diagnostics, Edison, NJ.
- <sup>e</sup> Thromboplastin LI, Helena Diagnostics, Beaumont, TX.
- <sup>f</sup> Russell's viper venom, American Diagnostica, Stamford, CT.
- <sup>g</sup> Oxoid Signal Blood Culture System Medium.
- <sup>h</sup> Idexx Laboratories, Westbrook, ME.

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