Optimization of coagulometric tests that incorporate human plasma for determination of coagulation factor activities in canine plasma

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Objective—To optimize methods used to measure coagulation factor activities in canine plasma, define reference ranges in dogs, and compare activities between canine and human plasma.

Sample Population—Human plasma samples (n = 5) and plasma from healthy dogs (140) and dogs with low factor V activity (7), high factor V activity (7), and low factor VIII:C activity (6).

Procedure—Coagulometric tests incorporated human plasma deficient in a single coagulation factor (human deficient plasma). Standard curves were generated with pooled plasma from 100 healthy dogs. Effect of sample dilution was evaluated, using plasma from dogs with high or low factor V activity and low factor VIII:C activity. Reference ranges for healthy dogs were established. Activities in human plasma were determined by comparison with standard curves obtained with canine plasma.

Results—Activities of factors V and VIII:C in samples diluted ≤ 1:20 influenced results of tests for other coagulation factors. Activities of factors V and VIII:C in human plasma were significantly less than in canine plasma. For the other coagulation factors, significant differences in human plasma-to-canine plasma activity ratios were detected among different sample dilutions.

Conclusions and Clinical Relevance—Accurate measurement of coagulation factor activities in canine plasma, using human deficient plasma, requires higher sample dilutions (ie, > 1:20) than typically used for human plasma. Differences in activities between human and canine plasma and nonparallelism of the standard curves emphasize the necessity for use of species-specific standard curves for accurate determination of coagulation factor activity. (*Am J Vet Res* 2001;62:625–629)

Determination of plasma activities of individual coagulation factors in dogs is important for the diagnosis and monitoring of hemostatic disorders such as hemophilia A and B.[1-3] In addition, measurement of coagulation factor activities in dogs is of interest in human medicine, because dogs may serve as an animal model to investigate questions concerning the coagulation system.[4-6]

Significant differences in coagulation factor activities are found between canine and human citrated plasma.[7-16] In general, activities of factors V and VIII:C are increased (6 to 13 times and 5 to 12 times, respectively) in dogs, compared with humans.[7,9,11-17] Despite this fact, activities of coagulation factors in canine plasma are typically determined coagulometrically, using commercially available artificial or congenital human deficient plasma (ie, plasma deficient in a specific factor) and test protocols optimized for human plasma.[11-16] Cats also have greater plasma factor V and VIII:C activities than humans, and protocols designed for use with human plasma have low specificity when used with feline plasma.[18] Therefore, the purposes of the study reported here were to optimize protocols of tests that incorporate human deficient plasma for determination of coagulation factor activities in canine plasma, to establish reference ranges for these activities, and to compare activities between human and canine plasma.

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Materials and Methods

Sample population—Citrated plasma for use in preparing canine pooled plasma and for determination of reference values was obtained from 140 healthy dogs from which food had been withheld for 12 hours. These dogs were between 1 and 8 years old and represented both sexes and various breeds. Dogs were determined to be healthy on the basis of clinical examination and results of CBC and serum biochemical analyses.

Additionally, 7 plasma samples with high factor V activity (> 200%) were obtained from dogs with hypercoagulability disorders, and 7 samples with low factor V activity (< 20%) were obtained from dogs with hypocoagulability disorders. Activities of other coagulation factors in these samples were minimally altered. Six plasma samples with low factor VIII:C activity (< 10%) were also obtained from dogs with hemophilia A; activities of the other coagulation factors in these samples were within reference ranges.

Collection of blood and preparation of platelet poor plasma—Blood samples were obtained from the cephalic or saphenous vein. The first 2 ml of blood were collected into tubes containing EDTA or lithium-heparin as the anticoagulant. These samples were used for CBC and serum biochemical analyses. Nine milliliters of blood were then collected into tubes containing 1 ml of sodium citrate solution[a] (0.11*M*) and mixed immediately. To obtain **platelet-poor plasma (PPP)**, blood samples were centrifuged at 2,000 × g for 20 minutes at 4 C. Plasma samples were stored in small aliquots (< 500 µl) at –70 C and thawed at 37 C immediately before determination of coagulation factor activities. Canine pooled plasma, prepared by mixing identical volumes of PPP from 100 healthy dogs, was also stored in small aliquots at –70 C.

Measurement of plasma activities of coagulation factors—Lyophilized human plasma deficient in factor II, V, VIII:C, IX, X, XI, or XII,[b] factor VII,[c] or **prekallikrein (PK)** or **high-molecular weight kininogen (HMWK)**[d] was acquired

from commercial sources and dissolved according to the manufacturers' instructions. Calcium-thromboplastin[e] and **activated partial thromboplastin time (APTT)** reagent[f] were also dissolved according to the manufacturers' instructions. All measurements were performed, using a 4-channel small-hook coagulometer.[g]

To determine the effects of sample dilution on results, activities were determined in plasma samples from healthy dogs and canine plasma samples with high (> 200%) or low (< 20%) factor V activity and low (< 10%) factor VIII:C activity that were diluted 1:5, 1:10, 1:20, 1:40, and 1:80 in **diethylbarbiturate-acetate (DBA)** buffer (pH 7.6).[c] On the basis of these results, plasma samples from healthy dogs were diluted 1:40 in DBA buffer prior to determination of reference values for individual coagulation factor activities.

To determine activities of factors II, V, VII, and X, 50 µl of the appropriate human deficient plasma were added to 50 µl of diluted PPP. Samples were incubated for 1 minute at 37C, and coagulation time was recorded after addition of 200 µl of calcium-thromboplastin. Calcium-thromboplastin was preincubated for at least 1 hour at 37C. To determine activities of factors VIII:C, IX, XI, and XII, PK, and HMWK, 100 µl of APTT reagent were added to a mixture of 50 µl of PPP and 50 µl of the appropriate human deficient plasma. After an incubation time of exactly 3 minutes at 37C, 100 µl of warm (37C) $CaCl_2$ solution (25 m*M*)[a] were added, and coagulation time was measured.

Recorded coagulation times for canine PPP and human plasma were transformed to percentage of activity by comparison with a standard curve. Standard curves were prepared for each coagulation factor and all charges of activating reagents by diluting canine pooled plasma with DBA buffer. Activity was considered to be 100% in canine pooled plasma tested at the same dilution as PPP. For example, canine PPP samples were diluted 1:40 for establishment of reference ranges. Activity in the standard diluted 1:40 was considered 100%, whereas activities in the 1:20 and 1:4,000 dilutions were considered 200 and 1%, respectively. Coagulation times in each dilution of standard were measured 5 times, and medians were calculated as the basis for the standard curve. Measurements of activities of coagulation factors in canine PPP and human plasma were performed in duplicate, and results were averaged.

Comparison of coagulation factor activities in canine and human plasma—Activities of coagulation factors were determined in 5 human reference plasma samples[h-l] at various dilutions (1:5 to 1:640), according to the protocol described for canine plasma. Coagulation times for human plasma were transformed to percentage of activity by comparison with a standard curve prepared with canine pooled plasma. If the dilution used was not 1:40, the percentage of coagulation factor activity in human plasma was adjusted. For example, results for human plasma diluted 1:80 were multiplied by 2. If activity in human plasma was not 100% (eg, 105%), an additional correction was made (eg, measured activity × 0.95).

Statistical analyses—Mean values (± SD) were calculated. Additionally, 2.5, 25, 50 (median), 75, and 97.5 percentiles were determined for reference values. Activities in plasma samples of various dilutions and activities in human and canine samples were compared by use of 1-way ANOVA and, when indicated, the *t*-test for combined samples. Significance was set at $P < 0.05$.

Results

Effect of sample dilution—In canine plasma with high (> 200%) or low (< 20%) factor V activity, sample dilution significantly affected activities of factors II and X (**Table 1**). In plasma with high factor V activity, activities of factors II and X were significantly greater in the 1:10 and 1:20 dilutions, compared with the 1:80 dilution, whereas in the 1:40 dilution, activities of factors II and X were not significantly different. The most striking divergence was detected for factor II activity in plasma with high factor V activity; mean activity determined for the 1:10 dilution was 1.38 times greater than that for the 1:80 dilution. In plasma with low factor V activity, factor II activity was significantly less for the sample diluted 1:10, compared with the 1:80 dilution, and factor X activity was significantly less for samples diluted 1:10 and 1:20.

Similar differences were detected for activities of factors IX and XII in canine plasma with low factor VIII:C activity (**Table 2**). Decreasing values of factor IX and XII activities were found with decreasing dilutions. For example, activities of factors IX and XII for the 1:5 dilution were only 68 and 69%, respectively, of activities for the 1:80 dilution. Sample dilution did not significantly affect activities of factors II, X, IX, or XII in

Table 1—Influence of sample dilution on mean ± SD activities of factors II and X in plasma from dogs with high (> 200%), low (< 20%), or normal (within reference range) factor V activity

Sample dilution	Factor II activity (%)	Factor X activity (%)
High (n = 7)*†		
1:10	105.6 ± 25.1‡	143.4 ± 43.9§
1:20	85.9 ± 23.1§	135.1 ± 41.8§
1:40	80.4 ± 19.8	127.4 ± 36.5
1:80	76.9 ± 19.6	125.9 ± 33.3
Low (n = 7)		
1:10	22.7 ± 7.9§	24.4 ± 10.7§
1:20	24.8 ± 9.3	25.8 ± 11.4§
1:40	25.5 ± 10.2	27.6 ± 12.6
1:80	26.4 ± 10.5	28.3 ± 13.2
Normal (n = 6)		
1:10	102.5 ± 14.7	94.0 ± 16.6
1:20	98.4 ± 10.2	93.6 ± 14.5
1:40	99.2 ± 11.2	91.8 ± 15.2
1:80	99.0 ± 11.7	93.3 ± 15.9

*Factor II activity in plasma with high factor V activity was significantly ($P < 0.001$; ANOVA) different among dilutions. †Factor X activity in plasma with high factor V activity was significantly ($P < 0.05$; ANOVA) different among dilutions. ‡Significantly ($P < 0.001$; *t*-test) different from value for sample diluted 1:80. §Significantly ($P < 0.05$; *t*-test) different from value for sample diluted 1:80.

Table 2—Influence of sample dilution on mean ± SD activities of factors IX and XII in plasma from dogs with low (< 10%) or normal (within reference range) factor VIII:C activity

Dilution	Factor IX activity (%)	Factor XII activity (%)
Low < 10% (n = 6)*†		
1:5	64.2 ± 16.1‡	59.8 ± 8.9‡
1:10	72.4 ± 14.5‡	67.4 ± 8.8§
1:20	79.6 ± 15.1‡	72.1 ± 9.9§
1:40	87.2 ± 18.6§	85.5 ± 4.6
1:80	92.9 ± 18.5	87.4 ± 6.2
Normal (n = 6)		
1:5	98.3 ± 18.9	102.3 ± 15.8
1:10	97.2 ± 13.7	102.5 ± 14.1
1:20	96.7 ± 20.1	101.0 ± 13.1
1:40	96.3 ± 22.3	101.4 ± 11.0
1:80	98.1 ± 22.0	103.4 ± 19.7

*Factor IX activity in plasma with low factor VIII:C activity was significantly ($P < 0.001$; ANOVA) different among dilutions. †Factor XII activity in plasma with low factor VIII:C activity was significantly ($P < 0.01$; ANOVA) different among dilutions. ‡Significantly ($P < 0.001$; *t*-test) different from value for sample diluted 1:80. §Significantly ($P < 0.01$; *t*-test) different from value for sample diluted 1:80.

Table 3—Reference values for activities of coagulation factors in canine plasma from healthy dogs determined by use of a coagulometric method, using human deficient plasma

Factor (n)	Mean ± SD†	Percentile*				
		2.5	25	50	75	97.5
II (138)	100.4 ± 12.2	80	92	100	108	126
V (136)	104.9 ± 21.0	75	92	100	114	158
VII (137)	101.2 ± 31.6	57	80	95	120	181
X (138)	99.5 ± 16.4	71	88	99	110	132
VIII:C (140)	99.7 ± 17.6	72	87	98	113	136
IX (138)	99.8 ± 16.2	75	88	99	110	140
XI (127)	99.4 ± 13.6	74	90	98	110	125
XII (128)	96.0 ± 15.0	73	85	95	107	127
PK (122)	100.4 ± 18.3	71	89	98	110	141
HMWK (122)	99.6 ± 15.7	80	89	96	109	142

*Reference range reported as values within the 2.5 to 97.5 percentile range. †Activities reported as percentage of activity in canine pooled plasma.
PK = Prekallikrein. HMWK = High-molecular weight kininogen.

Table 4—Mean ± SD activities, reported as percentage of activity in canine pooled plasma, of coagulation factors in human plasma (n = 5) determined by use of a coagulometric method, using human deficient plasma

Dilution of human plasma	Coagulation factor									
	II*	V	VII*	X†	VIII:C	IX†	XI‡	XII†	PK†	HMWK†
1:5	ND	11.3 ± 1.5	ND	ND	11.6 ± 2.1	ND	ND	ND	ND	ND
1:10	ND	10.0 ± 1.3	27.2 ± 1.8	ND	12.0 ± 3.4	44.8 ± 2.3	30.5 ± 2.8	45.2 ± 6.5	ND	46.0 ± 2.5
1:20	84.5 ± 8.9	10.8 ± 1.3	28.8 ± 3.2	79.7 ± 7.6	13.6 ± 1.9	57.3 ± 6.7	37.3 ± 6.3	55.7 ± 8.9	ND	61.5 ± 4.2
1:40	101 ± 9.3	11.3 ± 1.9	32.8 ± 2.0	103 ± 7.6	15.6 ± 2.1	70.3 ± 2.6	41.7 ± 6.8	81.5 ± 10.3	175 ± 21.3	85.2 ± 2.8
1:80	114 ± 13.1	10.8 ± 1.9	33.3 ± 2.9	121 ± 10.7	12.5 ± 2.6	86.1 ± 6.6	41.8 ± 8.6	95.5 ± 13.4	304 ± 20.9	129 ± 12.9
1:160	123 ± 15.4	10.3 ± 0.8	38.8 ± 5.2	144 ± 11.1	14.9 ± 4.3	94.5 ± 14.3	46.0 ± 4.4	109 ± 11.2	519 ± 25.4	140 ± 6.8
1:320	129 ± 14.7	ND	41.0 ± 8.6	153 ± 11.0	12.6 ± 3.8	117 ± 20.0	47.7 ± 6.1	122 ± 16.8	803 ± 73	157 ± 12.9
1:640	117 ± 26.4	ND	ND	163 ± 22.6	ND	110 ± 17.4	37.7 ± 6.0	141 ± 23.7	233 ± 79	175 ± 18.8

*Activity significantly ($P < 0.05$; ANOVA) different among dilutions. †Activity significantly ($P < 0.001$; ANOVA) different among dilutions. ‡Activity significantly ($P < 0.01$; ANOVA) different among dilutions.
ND = Not determined.

plasma samples in which factor V and factor VIII:C activities were within reference ranges.

Reference values—Reference ranges (ie, values within the 2.5 to 97.5 percentile range) were determined, using canine PPP diluted 1:40. The widest reference range detected was that for factor VII (**Table 3**).

Comparison of coagulation factor activities in canine and human plasma—Compared with the standard curve obtained with canine pooled plasma, median activities of factors V and VIII:C in human plasma were 10.8 and 12.6%, respectively (**Table 4**). In other words, activities of factor V and VIII:C in plasma from healthy dogs were 9.3 and 7.9 times, respectively, greater than activities in human plasma.

Activities of factors II, VII, X, IX, XI, and XII, PK, and HMWK in human plasma were significantly affected by sample dilution (Table 4). In more dilute samples, activities were increased, compared with the standard curve obtained with canine pooled plasma. Thus, ratios of coagulation factor activity in human plasma to activity in canine plasma could not be precisely determined. Activities of HMWK and factors II, X, IX, and XII in human plasma were within the range of activities in canine pooled plasma (ie, approx 100% of activity in canine plasma). However, activities of factor VII (27.2 to 41.0% of activity in canine plasma) and factor XI (30.5 to 47.7% of activity in canine plasma) were significantly less and activity of PK (175 to 1,233% of activity in canine plasma) significantly greater in human plasma.

Discussion

Results of this study indicate that methods to measure activities of individual coagulation factors in canine plasma, using human deficient plasma, are influenced by variations in activities of factors V and VIII:C. This is particularly true when low dilutions (ie, < 1:20) of canine plasma are assayed. Determination of activities of coagulation factors in human plasma is typically performed with plasma samples diluted ≤ 1:20.

Coagulometric tests allow determination of activity of a specific coagulation factor in an unknown plasma sample when the unknown diluted sample is mixed with undiluted plasma deficient in that specific factor. The specific factor is supplied by the unknown sample; all other factors are supplied by the deficient plasma. The test principle is based on the modified screening tests for **prothrombin time** (**PT**; factors II, V, VII, and X) or APTT (factors VIII:C, IX, XI, XII, PK, HMWK). The specificity of the test in humans results from the fact that variations in other factors in the unknown sample that may alter results of the modified screening tests do not cause essential variations in total activity of these factors in the test. However, plasma activities of factors V and VIII:C were 9.3 and 7.9 times greater, respectively, in plasma from healthy dogs, compared

with activities in human deficient plasma. Therefore, canine plasma at low dilutions (eg, 1:10) contribute to overall measured activities of factors V (a PT-dependent factor) and VIII:C (an APTT-dependent factors) and influence test results (**Fig 1**). In addition, to a lesser extent, alterations of factor VII activity, which was approximately 3 times higher in canine plasma than in human plasma, may contribute to this effect for PT-dependent factors. Use of higher sample dilutions (eg, 1:40) of canine plasma reduces this effect. Thus, canine plasma should be diluted > 1:20 to allow accurate measurement of individual coagulation factor activities by use of tests that incorporate human deficient plasma.

The results of Murtaugh and Dodds[19] as well as Mansell et al[20] also indicate that factor VIII:C activity influences activities of other coagulation factors of the intrinsic system. Again, this applies particularly when evaluating low dilutions of canine plasma. Thus, as measured by use of coagulometric tests that incorporate human deficient plasma, activities of factors IX, XI, and XII are low in plasma from dogs with markedly reduced factor VIII:C activity attributable to hemophilia A. Factor V activity is 5 times greater in feline plasma, compared with human plasma, and an interaction between factor V and factor II activities has also been documented when low dilutions of feline plasma are evaluated.[18]

The results of the present study are also of importance for other species with plasma activities of certain coagulation factors greater than those in humans. For example, activities of factors V and VIII:C are more than 5 times higher in pigs and cattle than in humans,[21] suggesting that more dilute samples should be assayed for these species as well.

In accordance with suggestions by Gross and Wichmann[22] and Ackermann,[23] reference ranges were established, using the distribution-independent 2.5 and 97.5 percentiles. The wide range in factor VII activity that was detected in the present study has been described in a previous study.[24] Besides breed-related differences in factor VII activity,[24] this is probably a result of the brief half-life of this coagulation factor.[25]

The marked increase in activities of factors V and VIII:C in canine plasma, compared with human plasma, corresponds well with results of other studies.[7,9,11-17] These high activities contribute to the decreased PT and APTT of dogs, compared with humans. Because of the influence of sample dilution on results of the other coagulation factor assays, ratios of activity in canine plasma to activity in human plasma could only be determined as ranges. The influence of sample dilution may also be a reason for the variable ratios reported by others.[7-17] Ratios reported in previous studies varied from 1.0[14] to 2.1[12] for factor II, 1.1[13] to 5[14] for factor X, 3[12-14] to 16[9] for factor IX, and 1.0[16] to 1.9[13] for factor XII. Moreover, in these studies, human rather than canine plasma was used to establish standard curves, and canine plasma was tested at only 1 dilution. The dilution used also varied from study to study. In one,[14] a 1:10 dilution was used for all tests, and in another,[16] a 1:5 dilution was used for APTT-dependent factors and a 1:20 dilution for PT-dependent factors. Any influence that type of plasma used to generate the standard curve has on comparisons of human and canine plasma was minimized in the present study by preparing canine pooled plasma with plasma from a large number of healthy dogs and testing human reference plasma from several different manufacturers.

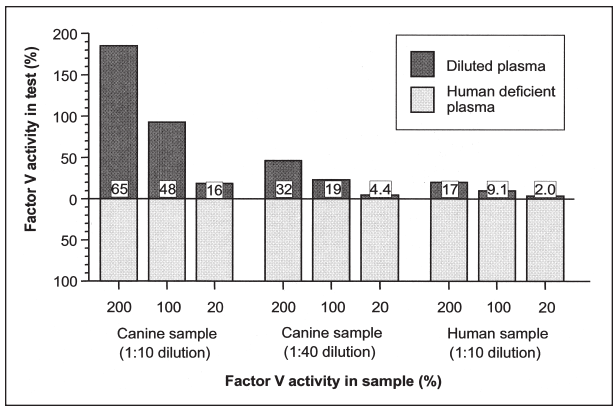

Figure 1—Effect of factor V activity in canine and human plasma samples on overall activity of factor V in coagulometric tests that use human plasma deficient in factor II, VII, or X (human deficient plasma) to measure activities of factors II, VII, or X, respectively. Activity of factor V in human plasma was considered to be 100%; activity in canine plasma was calculated to be 9.3 times greater than that in human plasma. Numbers within or on the bar graph indicate the percentage of factor V activity contributed by diluted plasma in relation to overall factor V activity in the test.

Sample dilution also influences the ratios of coagulation factor activity in feline or bovine plasma to activity in human plasma. Specific factors affected by sample dilution include factors IX and XII[26] and IX, XI, and XII[27] in feline plasma and factors XI and XII in bovine plasma.[21] Lutze and Kutschmann[26] as well as Lutze et al[21] compared different dilutions of feline and bovine pooled plasma with human reference plasma and found decreased activities in feline and bovine plasma with increasing sample dilution. This was reflected by a marked divergence of the corresponding standard curves. The high factor V and VIII:C activity in dogs, cats, and cattle certainly contributes to this phenomenon. Nevertheless, this cannot be the sole cause for the striking differences in coagulation factor activity, because activities varied even in more dilute samples (eg, 1:160, 1:320, and 1:640).

In the present study, PK activity was more varied between human and canine plasma than activities of the other factors. Because APTT in healthy dogs is less than in humans, Saito et al,[8] who reported that PK activity in canine plasma was 2% that in human plasma, suggest that low PK activity is an artifact attributable to species-specific differences in enzyme activities.

The differences in reference curves obtained with canine and human plasma, as demonstrated in the present study, indicate that calibration of tests that use standard curves derived from human plasma for determination of coagulation factor activities in canine plasma can only serve as an approximation under exactly defined conditions, especially concerning sample dilution. Creating a species-specific standard curve is, therefore, highly recommended. The disadvantages in using species-specific plasma to generate a standard curve, including a lack of stability or reproducibility,[26] can be

avoided by using a large number of samples and an appropriate choice of animals. The most important result of the present study, however, is that accurate determination of coagulation factor activities in canine plasma by use of coagulometric methods that incorporate human deficient plasma requires higher dilutions of canine plasma than those typically used for human plasma.

[a]Roche Diagnostics GmbH, Mannheim, Germany.
[b]Diagnostica Stago, Roche Diagnostics GmbH, Mannheim, Germany.
[c]Dade Behring Marburg GmbH, Marburg, Germany.
[d]Sigma Diagnostics, St. Louis, Mo.
[e]Thromborel S, Dade Behring Marburg GmbH, Marburg, Germany.
[f]Pathromtin, Dade Behring Marburg GmbH, Marburg, Germany.
[g]Schnitger & Gross coagulometer, Heinrich Amelung GmbH, Lemgo, Germany.
[h]Standard human plasma, Dade Behring Marburg GmbH, Marburg, Germany.
[i]CoagCal, Dade Behring Marburg GmbH, Marburg, Germany.
[j]STA factor calibrator, Roche Diagnostics GmbH, Mannheim, Germany.
[k]ACCUCLOT, Sigma Diagnostics, St. Louis, Mo.
[l]Reference plasma 100%, Immuno-Diagnostika, Progen Biotechnik GmbH, Heidelberg, Germany.

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