The importance of accurate citrate to blood ratios in the collection of canine blood for hemostatic testing

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The prothrombin time (PT), activated partial thromboplastin time (PTT), and thrombin clotting time (TCT) are laboratory tests frequently used in dogs to assess the integrity of the clotting mechanism, and to screen for coagulation disorders. Measurements of canine plasma Factor VIII (FVIII) activity and canine von Willebrand factor antigen (vWF:Ag) are probably the two most common specific hemostatic factor assays performed by veterinary hemostatic testing laboratories, because these determinations are used in the diagnosis of the two most prevalent inherited bleeding disorders in the dog, FVIII deficiency and von Willebrand's disease (1,2). The performance of any of the above tests is dependent on the laboratory receiving plasma from a properly collected and processed blood sample. The required anticoagulant for coagulation testing is trisodium citrate, and the plasma should be separated promptly from the cellular elements in order to avoid artifactual changes that may affect the above mentioned laboratory procedures (3,4).

Commercial blood collection tubes for coagulation testing (such as the Vacutainer system; Becton-Dickinson, Rutherford, New Jersey, USA) contain a premeasured volume of 3.8% sodium citrate anticoagulant into which blood is drawn by vacuum. When properly filled, the recommended ratio of one part anticoagulant to nine parts blood (vol/vol) is attained (5). This results in a final blood sample citrate concentration of 0.38%. In my experience, a common problem using vacuum-dependent blood collection systems is improper filling of the collection tube so that the expected 1:9 anticoagulant to blood ratio is not attained. Underfilling (possibly due to vacuum loss) is much more likely than overfilling. The former situation can predispose to unusually high citrate concentrations in the blood and, ultimately, in the plasma that is submitted for testing. Decreased citrate to blood ratios have been shown to significantly affect in vitro assessments of platelet function (6). Since most coagulation responses are also calcium dependent, in vitro assessment of coagulation parameters could be significantly affected by altered anticoagulant to blood ratios. In addition, because of the significant volume of anticoagulant present in coagulation collection tubes, dilutional artifacts can be introduced if tubes are improperly filled.

The purpose of the study reported herein was to determine the effects of altered citrate to blood ratios on coagulation screening tests and FVIII/vWF:Ag determinations, as performed on canine plasmas.

A 20 mL volume of blood was collected rapidly from each of 12 clinically healthy adult dogs into a plastic syringe following a clean venipuncture. The blood was distributed immediately into each of four calibrated plastic tubes containing a premeasured 0.5 mL volume of 3.8% trisodium citrate anticoagulant, to produce anticoagulant to blood ratios of 1:5, 1:7, 1:9, and 1:11. These mixtures corresponded to final blood citrate concentrations of 0.63%, 0.48%, 0.38%, and 0.32%, respectively. Each tube was capped and gently but thoroughly mixed. A microhematocrit determination was performed on each citrated blood mixture prior to centrifugation of the blood for platelet-poor plasma (PPP). Each PPP was stored in aliquots at −70°C prior to carrying out the various hemostatic tests (done within one week of collection).

Table 1. The effects of various blood to citrate ratios on the prothrombin time (PT), activated partial thromboplastin time (PTT), and thrombin clotting time (TCT) tests (mean ± 1SD; n=12)

Coagulation test	Anticoagulant to blood ratio			
	1:5	1:7	1:9	1:11
PT (s)	11.2 ± 2.1[a]	9.2 ± 1.3[a]	8.7 ± 1.3	8.4 ± 1.0
PTT (s)	18.1 ± 2.2[a]	16.6 ± 2.0[a]	15.9 ± 2.0	15.9 ± 2.1
TCT (s)	11.9 ± 1.9[a]	10.3 ± 1.5[a]	9.6 ± 1.2	9.2 ± 1.3

[a]Significantly different from the "standard" 1:9 mixture ($p<0.01$)

The PTT, PT, and TCT determinations were carried out as previously described (3). Factor VIII activity was quantitated using a one-stage differential partial thromboplastin time assay, and vWF:Ag concentrations were assayed using a Laurell electroimmunoassay (1). Factor VIII activity and vWF:Ag concentration were each expressed as a percentage of the activity/concentration in a normal canine reference plasma. All four plasmas derived from a single blood donation were tested at the same time under the same conditions. Plasma derived from the 1:9 anticoagulant to blood mixture (0.38% blood citrate concentration) was considered to be the "standard plasma", since this is the recommended anticoagulant to blood ratio (3–5). Hemostatic test results for each of the other plasmas were assessed in comparison to those for this "standard plasma" using Students's paired *t* test (7). A p value of <0.01 was considered to be statistically significant.

The hematocrit values (mean ± SD) for the 1:5, 1:7, 1:9, and 1:11 citrate to blood mixtures were 0.42 ± 0.04 L/L, 0.44 ± 0.05 L/L, 0.46 ± 0.05 L/L, and 0.47 ± 0.05 L/L, respectively. When compared to the "standard" 1:9 mixture, the hematocrit values are identical to the predicted mean hematocrit values for these mixtures, based on dilutional effects alone (0.42 L/L, 0.44 L/L, 0.46 L/L, and 0.47 L/L, respectively).

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Table 2. The effects of various blood to citrate ratios on plasma Factor VIII (FVIII) and von Willebrand factor antigen (vWF:Ag) determinations (mean ± 1SD; n=12)

	Anticoagulant to blood ratio			
Assay	1:5	1:7	1:9	1:11
Actual FVIII (%)[b]	71.6 ± 11.4[a]	83.2 ± 13.9[a]	88.3 ± 13.5	88.7 ± 13.3
Predicted FVIII[c] (%)	81.8	85.8	88.3	90.0
Actual vWF:Ag (%)[b]	99.5 ± 6.6[a]	108.3 ± 29.9[a]	115.7 ± 30.9	118.9 ± 32.5
Predicted vWF:Ag[c] (%)	107.1	112.5	115.7	117.9

[a]Significantly different from the "standard" 1:9 mixture ($p<0.01$)
[b]Percent of normal canine reference plasma
[c]Predicted mean value based on dilutional effects (compared to the "standard" 1:9 mixture)

The PT, PTT, and TCT results were all significantly prolonged by decreasing the citrate to blood ratio to 1:7 or 1:5. Increasing the citrate to blood ratio to 1:11 did not significantly effect any of the coagulation screening tests (Table 1).Plasma FVIII activity and vWF:Ag concentrations were both significantly reduced by decreasing the citrate to blood ratio to 1:7 or 1:5. The degree of reduction was more than could be accounted for on the basis of dilutional effects alone (Table 2). Increasing the citrate to blood ratio to 1:11 caused a slight increase in both FVIII activity and vWF:Ag concentrations (compared to the "standard" sample); however, these increases were not statistically significant ($p=0.82$ and $p=0.02$, respectively) and could be accounted for on the basis of the dilutional effect alone.

This study indicates that reducing the ratio of citrate to blood to below the recommended 1:9 can have significant effects on coagulation screening tests and on measurements of plasma FVIII activity and vWF:Ag concentrations. The PTT and PT are both dependent on recalcification of citrated plasma. A prolongation of clotting times might therefore be expected if citrate concentrations in the plasma were significantly increased (8). The plasma concentrations of citrate were obviously higher in the 1:5 and 1:7 anticoagulant blood mixtures than in the "standard" anticoagulant blood mixture (1:9). The TCT is a screening test that measures the rate of conversion of fibrinogen to fibrin by thrombin, a Ca^{++} independent step in the coagulation cascade. The TCT is, however, accelerated by the presence of Ca^{++}, since FVIII activation is enhanced by Ca^{++} and the clot can be detected more easily (4). Since the TCT is commonly performed without the addition of exogenous calcium, over-citration of plasma as a result of decreasing the citrate to blood ratio might be expected to slow the TCT, as happened in the present study.

Factor VIII activities were significantly lower in the plasmas derived from the 1:5 and 1:7 citrate blood mixtures than in the plasma derived from the "standard" 1:9 mixture. These reductions are more than can be accounted for by dilutional effects alone. Since the FVIII assay used is a differential partial thromboplastin time technique requiring recalcification of the reagent mixture, prolongations of the clotting time in over-citrated plasma samples might be expected. This would result in an underestimation of FVIII activity when measured against the canine reference plasma. Von Willebrand factor antigen concentrations were also significantly lower in the plasmas derived from the 1:5 and 1:7 citrate blood mixtures than in the plasma from the 1:9 mixture. This too may well reflect both dilutional effects and the effect of increased citrate concentrations on Ca^{++} availability. The rocket height in the electroimmunoassay can be affected by calcium concentrations (9). Reduced rocket height would result in a reduction in the calculated amount of vWF:Ag, when compared to the canine reference plasma.

None of the hemostatic tests studied was significantly affected by increasing the citrate to blood ratio from 1:9 to 1:11. This would suggest that slight overfilling of a blood collection tube is not of as great a concern as underfilling. In this study the final blood citrate concentration in the 1:11 mixture was 0.32%. A blood citrate concentration of 0.32% has been shown to be suitable for coagulation testing (5,10).

The present study suggests that underfilling citrated blood collection tubes containing a premeasured amount of citrate anticoagulant can have significant effects on a number of common hemostatic laboratory tests: clotting times may be significantly prolonged and factor levels significantly underestimated. It is important, therefore, that one ensures that blood collection tubes are properly filled and that the recommended 1:9 anticoagulant to blood ratio is attained. Since most commercial blood collection tubes are not calibrated, an accurate citrate to blood ratio can be best assured by removing the anticoagulant from the collection tube and putting it into a syringe. The anticoagulant to blood

ratio can then be monitored easily as the blood is collected.

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References

1. Johnstone IB. Classical haemophilia (Haemophilia A) in German shepherd dogs: Different expressions of the disease. Aust Vet Pract 1987; 17: 71–75.
2. Meyers KM, Wardrop KJ, Meinkoth J. Canine von Willebrand's disease: Pathobiology, diagnosis, and short-term treatment. Compend Contin Educ Pract Vet 1992; 14: 13–23.
3. Smalko D, Johnstone IB, Crane S. Submitting canine blood for prothrombin time and partial thromboplastin time determinations. Can Vet J 1985; 26: 135–137.
4. Tvedten H. Hemostatic abnormalities. In: Willard MD, Tvedten H, Turnwald GH, eds. Small Animal Clinical Diagnosis by Laboratory Methods. Philadelphia: WB Saunders, 1989: 86–102.
5. Becton Dickinson Vacutainer Systems-Product catalogue. Becton Dickinson and Company, 1981: 24.
6. Ts'ao C-H, Lo R, Raymond J. Critical importance of citrate-blood ratio in platelet aggregation studies. Am J Clin Pathol 1976; 65: 518–522.
7. Altman DG. Practical Statistics for Medical Research. London: Chapman and Hall, 1991: 179–276.
8. Greene CE, Tsang CW, Prestwood AK. Coagulation studies of plasmas from healthy domesticated animals and persons. Am J Vet Res 1981; 42: 2170–2177.
9. Laurell CB. Electroimmunoassay. Scand J Clin Lab Invest 1972; 29: 21–37.
10. Koepke JA, Rodgers JL, Ollivier MJ. Pre-instrumental variables in coagulation testing. Am J Clin Pathol 1975; 64: 591–596.

Answers to Quiz Corner/Les réponses du Test Éclair

1. e — Laryngeal paralysis is common in older Labrador retrievers. Affected dogs characteristically show upper airway obstruction that causes noise known as stridor.
   e — La paralysie laryngée est fréquente chez les vieux chiens de race Labrador retriever. Les animaux affectés montrent de façon caractéristique une obstruction des voies respiratoires supérieures qui cause un bruit connu sous le nom de stridor.

2. b

3. e

4. c

5. e

6. d

7. e — Intraosseous catheters provide the best access to the peripheral circulation of severely dehydrated birds.
   e — Les cathéters intraosseux constituent la meilleure voie d'accès à la circulation périphérique chez les oiseaux fortement déshydratés.

8. e — "Feline endocrine alopecia" is a misnomer. There is no documented hormonal excess or deficiency. Many cases of symmetric alopecia are caused by pruritus, while others are a behavioral "overgrooming" disorder.
   e — "L'alopécie endocrinienne féline" est une mauvaise appellation. Il n'y a pas d'excès ou de carence hormonale de rapporté dans la littérature. Plusieurs cas d'alopécie symétrique sont causés par du prurit alors que d'autres résultent d'un trouble comportemental "d'excès de toilettage".

9. c — Pulpal tissue exposed for several weeks is infected and necrotic. It should be removed and the cavity filled with inert material, or the tooth should be extracted.
   c — Le tissu pulpaire exposé à l'air pendant plusieurs semaines s'infecte et se nécrose. Il doit être enlevé et la cavité remplie avec une substance inerte ou la dent doit être extraite.

10. b — Pings associated with cecal torsion and abomasa volvulus are usually heard in the right paralumbar fossa. Pings associated with rumen gas are usually heard over the entire left side of the abdomen. Pings associated with pneumoperitoneum are usually detectable on both sides of the abdomen.
    b — Les bruits de "ping" associés à une torsion du caecum et à un volvulus de la caillette sont habituellement audibles dans la fosse paralombaire droite. Les bruits de "ping" associés à une accumulation de gaz dans le rumen sont habituellement audibles sur toute l'étendue du côté gauche de l'abdomen. Les bruits de "ping" associés à un pneumopéritoine sont habituellement décelables des deux côtés de l'abdomen.