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Prothrombin time and activated partial thromboplastin time reference intervals in dogs, by gender and age group using the Start®4 (Stago)

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ABSTRACT: *Prothrombin time (PT) and the activated partial thromboplastin time (aPTT) are useful tools for the diagnosis and monitoring of coagulation disorders in Veterinary Medicine. Our objectives were: to establish reference intervals (RI) for PT and a PTT for the dog using the Start®4 (Stago), to compare the obtained RI with literature; to evaluate the effects of gender and age on the coagulation profile. Plasma samples of 122 healthy dogs (57 males; 65 females) aged between 4 months and 18 years, divided into three age groups (0-2 years old; 3-10 years old; > 10 years old) and grouped in to males and females were analysed. The RI were estimated following the ASVCP guidelines with the Reference Value Advisor software. The RI were: PT 6.7" to 10.8"; aPTT 9.0" to 14.8". PT was significantly higher in females than in males. Dogs aged 10 years or older have significantly higher mean aPTT times than younger dogs. RI comparison showed a considerable percentage of cases outside the reference RI of the literature (PT - 79.3%; aPTT - 77.1%), demonstrating the need of each laboratory to calculate its own RI. The RI established in this study are applicable for the coagulation profile assessment in dogs.*
Key words: *PT, aPTT, reference intervals, dog, coagulation profile, Start®4.*

Intervalos de referência para o tempo de protrombina e tempo de tromboplastina parcial ativada no cão de acordo com a idade e o gênero utilizando o Start®4 (Stago)

RESUMO: *O tempo de protrombina (TP) e o tempo de tromboplastina parcial ativada (TTPa) são ferramentas úteis para o diagnóstico e monitorização das alterações da coagulação em Medicina Veterinária. Os objetivos deste estudo foram: estabelecer intervalos de referência (IR) para TP e TTPa para o cão utilizando o Start®4 (Stago), de modo a comparar os IR obtidos com a literatura; avaliar os efeitos do sexo e da idade no perfil da coagulação. Foram usadas amostras de plasma de 122 cães saudáveis (57 machos; 65 fêmeas) com idades entre quatro meses e 18 anos, divididos em três grupos (0-2 anos; 3-10 anos; > 10 anos) e agrupados em machos e fêmeas. Os IR foram calculados seguindo as diretrizes da ASVCP com o software Reference Value Advisor. Os IR obtidos foram: PT 6,7 '' a 10,8 ''; TTPa 9,0 '' a 14,8 ''. O TP foi significativamente maior nas fêmeas do que nos machos. Os cães com 10 anos ou mais apresentaram tempos médios de TTPa significativamente maiores do que cães mais jovens. A comparação de IR mostrou uma percentagem considerável de casos fora do IR de referência da literatura (TP - 79,3%; TTPa - 77,1%), confirmando a necessidade de cada laboratório calcular seu próprio IR. Os IR estabelecidos neste estudo são aplicáveis na avaliação do perfil hemostático em cães.*
Palavras-chave: *TP, TTPa, intervalos de referência, cão, perfil hemostático, Start®4.*

INTRODUCTION

Coagulation profile is a useful tool for the diagnosis of coagulation disorders and for monitoring anticoagulant therapy (HERRING & MCMICHAEL, 2012). In Veterinary Medicine the prothrombin time (PT) and the activated partial thromboplastin time (aPTT) performed in citrated plasma are the most commonly employed in patients with a coagulopathy suspicion (HERRING & MCMICHAEL, 2012).

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PT is a screening test for the extrinsic and common pathways (coagulation factors II, V, VII and X), whilst aPTT is a screening test for the intrinsic and common pathways (coagulation factors II, V, VIII, IX, X, XI and XII) (WINTER, 2017).

Reference intervals (RI) are basic tools used in medicine to interpret patient laboratory test results, allowing healthy and unhealthy individuals' differentiation (BONAR et al., 2017). The traditional approach is to compare the obtained laboratory results with the existing RI. However, the choice of adequate RI is essential for laboratories to provide reliable information and for Veterinarians to correctly understand the results (FRIEDRICHS et al., 2012). Regarding to canine hemostasis, GEFFRÉ and collaborators (2011a) recommend the determination of the laboratory's own reference intervals instead of validation of a pre-existing reference interval.

Reference intervals are known to be influenced by several factors such as age, gender, as well as breed or lifestyle (FRIEDRICHS et al., 2012; CERIOTTI, 2007). In the literature there are several reports on RI for PT and aPTT; however, none assess the influence of gender and age (GEFFRÉ et al., 2010; YANG et al., 2018) and used reagents and methodology are variable. Thus, this study's objectives were: to establish the internal RI for PT and aPTT for the dog using the Start®4 (Stago), a standard coagulation device; to compare the RI with previously published RI; to evaluate the effects of gender and age on the coagulation profile.

MATERIALS AND METHODS

Study population

This study included 122 samples from dogs that underwent a coagulation profile at the Clinical Pathology Laboratory of the University of Trás-os-Montes e Alto Douro Veterinary Teaching Hospital from March 2016 to August 2019. To study the effect of age and gender on PT and aPTT values the animals were divided into three age groups (0-2 years old; 3-10 years old; > 10 years old) (EPSTEIN et al., 2005) and grouped in to males and females, information on the reproductive status of the dogs was not collected.

Only animals considered healthy based on clinical history (with no history of disease in the last 6 months) and a thorough physical examination, as well as on the results of a complete blood count and biochemical analysis, namely liver profile (aspartate aminotransferase, alanine aminotransferase and alkaline phosphatase) and kidney profile (creatinine, phosphorus and urea) were included. Animals that received any medication in the previous 6 months, as well as pregnant and lactating bitches were excluded. The study was ethically approved by the board of the University of Trás-os-Montes e Alto Douro Veterinary Teaching Hospital as complying with the Portuguese legislation for the protection of animals (Law no. 92/1995, September 12), and followed the ASVCP guidelines for RI creation (FRIEDRICHS et al., 2012).

Sample collection and analysis

The samples were obtained by clean venipuncture (jugular vein, or cephalic vein) and immediately placed in 0.5 ml tubes with 3.8% sodium citrate (FL medical) respecting the 9:1 ratio of blood to citrate. No blood samples were collected exclusively for this study. The plasma was separated through routine centrifugation (800 g for 10 min). According to laboratory standards, strongly hemolyzed or lipemic samples were rejected. The plasma samples were analysed using Start®4 (Stago) following the manufacturer's instructions until 4 hours after collection. In every run, a control sample (Coag Control N + P - Stago®) was included. The measurement principle of PT and aPTT is based on the viscometric detection of clot formation. The clot detection is based on the increased viscosity of the plasma sample. The change in plasma viscosity is analyzed by measuring the amplitude of the movement of a bead through the action of two electromagnets activated alternately to induce a natural oscillation. The sample viscosity increases as the clot forms, with consequent decrease in the amplitude of the bead movement. The stopwatch stops when a clot forms, even in the presence of a weak clot and when the ball is not stopped. Using several algorithms PT and aPTT are calculated. This technique allows the use of hemolised, lipemic, or hiperbilirrubinemic samples.

Statistical analysis

The descriptive analysis and calculations of RI for PT and aPTT were performed using the program Reference Value Advisor - v2.1. It is a macro to calculate RI with Microsoft Excel, available on <http://www.biostat.envt.fr/reference-value-advisor/> (GEFFRÉ et al., 2011b).

The normality of the data was assessed using the Anderson-Darling test and the presence of outliers using the Tukey method, both implemented in the Reference Value Advisor. For estimating the RI, the ASVCP guidelines (FRIEDRICHS et al., 2012) were followed, namely with regard to the sample size

(between 40 and 140 cases). However, the number of dogs in the subsamples from 0 to 2 years and 10 years or older is less than the minimum recommended. For the estimation of the RI (90%), the parametric or robust method was used, with or without Box-Cox transformation, depending on the data distribution.

The analysis of the significance of the differences by gender and by age group was performed with the Student's T-Test and with the Analysis of Variance (ANOVA), respectively. In the cases where ANOVA was significant, the Tukey HSD multiple comparison test was used. These analyses were performed with the IBM SPSS - version 24 for Windows. A level of significance of 5% ($P < 0.05$) was considered.

In order to determine whether our results differ from those previously published in the literature: PT (6.4 to 7.4'') and aPTT (9 to 11'') (FELDMAN, 1986), we compared both. According to the ASVCP reference interval guidelines, the acceptance criterion was as follows: after the elimination of outliers, less than 10% of the results fell outside the literature PT and aPTT established RI (FRIEDRICHS et al., 2012).

RESULTS

This study included 122 dogs (57 males and 65 females) aged between 4 months and 18 years, with mean age of 6.4 years (SD = 4.5). Most dogs were mixed breed (70.5%). Sample characteristics regarding gender, age and breed are shown in table 1.

Table 2 shows the descriptive statistics and the reference values of the TP and the aPTT in the total sample, by gender and by age group.

Results in table 3 show that the PT is significantly higher in females (M = 8.5; SD = 1.1) than in males (M = 8.0; SD = 0.8) ($P = 0.015$). There were no statistically significant differences among the 3 age groups ($P = 0.740$).

Regarding the aPTT, there are no significant differences by gender ($P = 0.939$), but

Table 1 - Sample characteristics concerning gender, age and breed (N = 122).

Variables		N	%
Gender	Male	56	46.3
(N = 121)	Female	65	53.7
Age	0-2 years	29	27.1
(N = 107)	3-10 years	55	51.4
	10+ years	23	21.5
Minimum - maximum	0.33 - 18 years		
Mean (SD)	6.4 (4.5) years		
Breed	Mixed breed	86	70.5
(N = 122)	Labrador	6	4.9
	Boxer	5	4.1
	Yorkshire terrier	4	3.3
	German Shepherd	4	3.3
	Pinscher	3	2.5
	Beagle	2	1.6
	French Bulldog	2	1.6
	Serra da Estrela dog	2	1.6
	Shih-tzu	1	0.8
	Chihuahua	1	0.8
	Cocker	1	0.8
	Shar-pei	1	0.8
	Pug	1	0.8
	Zwergspitz	1	0.8
	Podengo	1	0.8
	Bouvier bernois	1	0.8

Table 2 - Prothrombin Time (PT) and Activated Partial Thromboplastin Time (aPTT) reference values.

	Minimum - maximum	Median	Mean	Standard Deviation	Reference Interval (RI)	-Confidence Intervals (90%)-	
						Lower limit	Upper limit
----------PT (seconds)----------							
Total (n = 121)	6.5 -10.8	8.0	8.3	1.0	6.7 – 10.8	6.6 – 6.9	10.3 – 11.4
Male (n= 56)	6.6 –10.3	7.9	8.0	0.8	6.8 – 9.9	6.7 – 7.0	9.4 – 10.4
Female (n = 64)	6.5 –10.8	8.2	8.5	1.1	6.0 – 10.8	5.6 – 6.4	10.2 – 11.2
0-2 years (n = 29)	6.6 – 10.8	7.8	8.1	1.1	6.6 – 11.4	6.4 – 6.9	9.9 – 14.0
3-10 years (n = 55)	6.6 – 10.7	8.0	8.2	1.0	6.8 – 10.7	6.6 – 7.0	10.0 – 11.5
10+ years (n = 23)	6.5 – 10.1	8.2	8.3	1.0	6.3 – 10.4	5.7 – 6.9	9.8 – 11.0
----------aPTT (seconds)----------							
Total (n = 122)	7.6 – 16.6	11.7	11.9	1.5	9.0 – 14.8	8.6 – 9.4	14.2 – 15.3
Male (n = 56)	7.6 – 16.6	11.7	11.9	1.7	8.3 – 15.1	7.6 – 9.1	14.4 – 15.9
Female (n = 65)	8.1 – 16.0	11.7	11.9	1.3	9.2 – 14.3	8.6 – 9.7	13.7 – 14.9
0-2 years (n = 28)	9.9 – 15.0	11.6	11.7	1.0	9.6 – 13.9	9.0 – 10.1	13.3 – 14.4
3-10 years (n=55)	7.6 – 16.6	11.6	11.6	1.4	8.8 – 14.6	8.1 – 9.5	13.8 – 15.2
10+ years (n =23)	9.9 – 16.2	12.3	12.6	1.5	9.0 – 15.6	8.2 – 10.1	14.3 – 16.7

NOTE: an outlier in the total PT and an outlier in the aPTT in the subsample from 0 to 2 years were excluded.

there are differences among age groups (P = 0.012). Multiple comparison tests show that dogs aged 10 years or older (M = 12.6; SD = 1.5) have significantly higher mean times (P < 0.05) than dogs aged 0 to 2 years (M = 11.7; SD = 1.0) and than dogs aged 3 to 10 years (M = 11.6; SD = 1.4). There were no significant differences between the two younger age groups (P > 0.05).

When comparing our RI with those in the literature (FELDMAN, 1986), after excluding the outliers, the results showed a considerable percentage of cases outside the reference RI found in the literature: 79.3% for PT and 77.1% for aPTT (Table 4).

DISCUSSION

The use of inappropriate RI may lead to results misinterpretation causing erroneous diagnosis and treatment (FRIEDRICHS et al., 2012). In this study, the PT and aPTT of 122 healthy dogs (65 females and 56 males) aged between 4 months and 18 years were analyzed with a viscosity-based detection system (Start®4 - Stago). There are several studies in the literature addressing RI for coagulation parameters in dogs; however, less are those that distinguish between males and females and age groups (FELDMAN, 1986; RIZZO et al., 2008; HERRING & MCMICHAEl, 2012), and as it is known RI vary between laboratories because reagents and methodology used are variable

As for our results, the RI for the PT vary from 6.7 to 10.8 seconds; and as for aPTT, ranged from 9 to 14.8 seconds. Also while for PT, significant differences were observed between genders and no differences detected between the age groups, for aPTT there were no differences between genders, but oldest age group (>10 years) presented the higher values. The effect of age on coagulation times, namely for aPTT was recently described in humans (AHMED et al., 2019) but no similar data was described for veterinary medicine. Disturbance in hemostasis can be a complication in dogs with heart disease. Dogs over 10 years included in this study had no physical examination findings indicative of the presence of heart disease, such as a murmur or an arrhythmia, and had no clinical signs consistent with the presence of congestive heart failure, such tachypnea, dyspnea, and/or respiratory distress, exercise intolerance, weakness and inappetence. Also in a study carried out by PRIHIRUNKIT et al. (2014) no statistically significant differences in aPTT were reported between the congestive heart failure dogs with mitral valve disease and the control group. Our results agree with previous statements, the partitioning of reference population into subclasses such as age and gender has

Table 3 - Comparison of PT and aPTT by gender and age group.

	PT M (SD)	aPTT M (SD)
----------Gender----------		
Male	8.0 (0.8)	11.9 (1.7)
Female	8.5 (1.1)	11.9 (1.3)
Student's T-Test	P = 0.015	P = 0.939
----------Age----------		
0-2 years	8.1 (1.1) [a]	11.7 (1.0) [a]
3-10 years	8.2 (1.0) [a]	11.6 (1.4) [a]
10+ years	8.3 (1.0) [a]	12.6 (1.5) [b]
ANOVA	P = 0.740	P = 0.012

[ab]there are no statistically significant differences between the age groups with the letter in common: P > 0.05 in the Tukey HSD multiple comparison tests.

decrease biological variability between individuals resulting on more accurate RI (WALTON, 2001).

When comparing our results with previously published RI (FELDMAN, 1986), more than 10% of the results fell outside the literature intervals. It is relevant to note that the study used to results comparison was produced with distinct methodologies and reagents. This is the reason why each laboratory should create internal RI. Otherwise, some errors with serious clinical implications may be inferred.

To be useful, RI should have origin in a similar animal population and be collected under similar pre-analytical conditions as those used by the adopting laboratory (HOROWITZ et al., 2008; FRIEDRICHS et al., 2012). For RI calculation, in veterinary Medicine, may be difficult to obtain a quality sample since there are several variables that significantly affect the sample and the number of samples available (CERIOTTI, 2007). The literature states that, for more accuracy, must be used a sample number greater than 120 in order to determine reference limits by nonparametric methods with 90% confidence intervals. (FRIEDRICHS et al., 2012, CERIOTTI, 2007). In our retrospective study, information on the clotting times of 223 dogs was obtained. After applying the established inclusion and exclusion criteria, the final sample was of 122 animals. The need of use healthy animals with no signs of disease in the 6 months prior sampling limited the number of samples to be used. Also the regularity which routine tests are performed in healthy animals is relatively low, when comparing with Human Medicine (FRIEDRICHS et al., 2012; FERREIRA & ANDRIOLO, 2008).

The literature recognizes the influence that sample collection, transport and storage have on the variability of the results obtained in this type of study. These procedures are categorized in the

Table 4 - Cases inside and outside the literature PT and aPTT reference intervals.

	PT – literature* (6.4" to 7.4")	APTT – literature* (9" to 11")
Below the lower limit	n = 0 (0.0%)	n = 3 (2.5%)
Normal (inside the reference RI)	n = 25 (20.7%)	n = 28 (22.9%)
Above the upper limit	n= 96 (79.3%)	n= 91 (74.6%)
Outside the reference RI	n = 96 (79.3%)	n = 94 (77.1%)

Note: One outlier was excluded for PT. *(FELDMAN, 1986).

pre-analytical phase, being associated with normal sample manipulation. Their elimination or mitigation is one of the greatest challenges for laboratories, and a standardisation of these procedures should always be followed. (MAGNETTE et al., 2016; MEINKOTH & ALLISON, 2007; McCRAW et al., 2010). To minimize the influence of pre-analytical factors on results, the Institute for Clinical and Laboratory Standards (CLSI) and the World Health Organisation have developed guidelines of good practice that laboratories must follow to maintain quality and validity of the service, as well as to assist those requesting the tests (LINSKENS & DEVREESE, 2018; JACOBSEN et al., 2018). In order to create reliable RI all the recommendations should be followed. The moment of sample collection is one of the most important pre-analytical factors. The ratio of blood to citrate should be respected, if tubes are under-filled, the relative excess of anticoagulant in the sample, could potentially prolong the PT and aPTT results (MEINKOTH & ALLISON, 2007). Coagulation factors are readily activated by venipuncture and are unstable in stored samples. A sample obtained by a traumatic venipuncture may have contamination with tissue factor. This contamination of samples obtained in citrated plasma may falsely reduce the aPTT; however, the effect will be lesser in the PT. A clean venipuncture should be promoted, the excitation of the animal at the time of blood sampling must be minimized, the animal must be quiet and at rest to avoid stress (MEINKOTH & ALLISON, 2007; McCRAW et al., 2010). In our study, in order to minimize these problems, the animals were kept as calm as possible and the blood collection tubes properly filled.

Mild prolongations in PT and aPTT results are observed in animals with an increased hematocrit. If an animal has a markedly increased hematocrit, there is less plasma volume which again results in a relative excess of anticoagulant. (MEINKOTH & ALLISON, 2007). In our study only animals with normal hematology were enrolled.

CLSI guidelines state that whole blood or plasma samples, stored at room temperature, for routine hemostasis tests or determination of clotting factors should be analysed within 4 hours after sample collection, with the exception of the TP that has a stability of up to 24 hours (RIZZO et al., 2008, PICCIONE et al., 2010). The plasma must be separated from the cells by centrifugation within 30 minutes after collection. A plasma kept in a 4 ºC refrigeration is generally stable for 48 hours (FURLANELLO et al., 2006). If hemostasis tests are to be carried out in a laboratory far from the sample collection site, the plasma should be frozen quickly and kept frozen during transport (LINSKENS & DEVREESE, 2018). Slow freezing or thawing cycles promote the formation of ice crystals and the precipitation of clotting factors. (MEINKOTH & ALLISON, 2007). In our study, samples were analyzed until 4 hours after collection.

CONCLUSION

In conclusion, the PT and aPTT reference values established in this study represent valuable and applicable ranges for the coagulation profile assessment in dog when using the Start®4 (Stago) within a similar population. As for gender and age, PT was higher in females and aPTT was higher in the oldest animals. Results also demonstrated the need of each laboratory to create its own RI. That has particular relevance for PT and aPTT as a small difference might change a medical or surgical decision.

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DECLARATION OF CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHORS' CONTRIBUTIONS

Barbosa, M performed the laboratory analysis, organized the database, the literature search and wrote the first draft of the article; Pires, MJ designed the study and reviewed the article; Queiroga, F reviewed and edited the article; Pires, C performed the statistical analysis; Dinis, T laboratory analysis and organized the database; Silvestre-Ferreira designed the study, performed the literature search and wrote the draft article. All authors, read, and approved the submitted version.

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