

Research Article

Establishment of Reference Ranges for Coagulation Tests for Dogs in Sri Lanka

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- Prothrombin time (PT); Activated partial thromboplastin time (aPTT); Clotting time (CT)
- Bleeding time (BT); Dogs; Reference ranges

Abstract

Coagulopathy is an important, common systemic clinical syndrome caused by snake envenoming in the world. The reference ranges, so far, used in the Veterinary Teaching Hospital (VTH) for clinical evaluation of dogs with coagulation abnormalities were from western countries which not the ideal is considering the geographical differences. Therefore, in this study have been established reference intervals for coagulation tests (PT, aPTT, CT, and BT) for dogs in Sri Lanka. Selection of dogs for suitable blood samples was done during January to June in 2012. Apparently healthy dogs that were brought to VTH for routine medical attention, routine general check-up, vaccination, and routine elective surgical interventions i.e. ovariohysterectomy or castration, were used for this purpose. First, a general clinical examination was performed on each selected dog and those who qualified were blood sampled for laboratory tests namely, Full Blood Count (FBC), Total Protein (TP), Albumin (Alb), Fibrinogen, Alanine aminotransferase (ALT), and Aspartate aminotransferase (AST). The blood samples from these 45 dogs were used to establish reference ranges for PT, aPTT, CT and BT. At the end of the period, 45 dogs were selected which were clinically healthy, with normal FBC and liver function test values. The reference intervals obtained using dogs for PT, aPTT, CT and BT were 7-11 seconds, 11-22 seconds, 3-12.5 minutes and 0.5-5 minutes, respectively. These values are of extreme importance in order to treat, manage and monitor snake envenomed dogs better.

ABBREVIATIONS

ANOVA: Analysis of Variance; SEM: Standard Error of Mean

INTRODUCTION

Haemostasis is the mechanism in operation in the body to stop bleeding and maintain blood in the fluid state within the vascular system [1]. There are at least four major systems involved in this complex process namely; the vascular system, platelets, fibrin forming system and the fibrinolysis system. Haemostasis consists of a tightly controlled and well balanced interplay among a large number of cellular and protein components. The endothelial cell lining of the blood vessels and platelets play an important role in this process. The generation of a blood clot is meticulously controlled through the complex interplay of the initial trigger, amplification and inhibition [2]. The second most numerous circulating components in blood are platelets and they are essential for blood coagulation, maintenance of vascular integrity and control of haemostasis. Platelets are the first line of defence against bleeding. They circulate for approximately 5-9 days in blood of most mammalian species [3]. Thrombocytopenia is defined as a decrease in circulating platelets and is the most common acquired haemostatic disorder in veterinary and human medicine. Thrombocytopenia can result from the decreased or defective platelet production, increased peripheral platelet loss or consumption and platelet destruction or abnormal distribution

[4]. Performing a coagulation screen is suggested in any animal where a coagulopathy is suspected. PT, aPTT, and platelet count are the minimum recommended laboratory tests to assess haemostasis in small animals (mouse or rat) and in large animals (dog or monkey) [5]. The assays are functional tests configured with specific reagents that sequentially activate distinct series of coagulation factors (i.e. PT and aPTT) [6]. In addition to those tests, when there is coagulopathy disorder due to snake bite envenomation, CT is measured. It is a simple, fast, cost effective and an easily performed test [7]. When there is a suspicion of coagulopathy with the history of snakebite envenomation, it is recommended to perform PT, aPTT, and CT in human hospitals in Sri Lanka [7]. The common pathway leading to clot formation is activated by the intrinsic and/ or extrinsic pathways [8]. The ability of a platelet to adhere aggregate and for the primary hemostatic plug is measured using BT and prolongation of this *in-vivo* method is indicted by thrombocytopenia [9]. Measurement of PT and aPTT is performed using citrated plasma, and they are the most commonly employed tests in human patients with a suspected coagulopathy [8,10]. The PT test is performed by adding tissue thromboplastin or tissue factor and Ca²⁺ to citrated plasma and measuring the time for clot formation. The PT reagent used in the testing provides the tissue thromboplastin and Ca²⁺ [6,11]. Snake envenomation has been a frequently reported acquired coagulation disorder in dogs in Sri Lanka, because it

causes abnormal bleeding due to vascular endothelial damage [12]. Snake venoms may produce local tissue damage or distinct clinical syndromes, including neurotoxicity and coagulopathy [13]. There are no currently available standard reference intervals for coagulation tests, including PT, aPTT, CT, and BT for dogs in Sri Lanka. Therefore, this study aims to establish reference values for PT, aPTT, CT, and BT in order to enable accurate diagnosis, treatment and management of snakebite envenomation in dogs.

METHODOLOGY

Selection of dogs

Data were collected on dogs (n= 196) subjected to admitted to the Veterinary Teaching Hospital (VTH), Peradeniya during a period of 06 months from January to June in 2012. Apparently healthy dogs that were brought to VTH for routine medical attention, routine general check-up, vaccination, and routine elective surgical interventions i.e. ovariohysterectomy or castration, were used for sampling following General Clinical Examination (GCE) and laboratory tests (Full Blood Count (FBC), Total Protein (TP), Albumin (Alb), Fibrinogen (Fib), Alanine aminotransferase (ALT), and Aspartate aminotransferase (AST). GCE was performed on all such selected dogs, including body temperature, heart rate and pulse rate and quality, respiratory rate and quality and colour of the mucosal membrane. Examination of cardiovascular system, integumentary system, lymphatic system, head and neck, respiratory system, digestive system and associated organs, urinary system, reproductive system, musculoskeletal system and nervous system were also included. Consent from the client was obtained. Dogs that showed any clinical abnormality or altered test results were considered as outliers, and removed from the study (Figure 1).

Collection of blood samples

5ml of blood were collected from each dog separately by an atraumatic venepuncture on the first attempt, allowing a free flow into the syringe avoiding unnecessary vacuum to prevent activation of platelets by turbulence. Either the cephalic vein or the saphenous vein was selected to collect blood samples with the aid of a hypodermic syringe and a hypodermic needle (21G). Blood was stored immediately after collection in three different tubes: (A). 2.5mL of blood was placed in a commercially prepared standard plastic tube which contain EDTA concentration of 1.5-2mg/mL (APTACA, Canelli-Italy), (B). 0.9mL of blood was placed in a tube with 3.2% (0.109 mol/L) tri-sodium citrate (anticoagulant to blood ratio of 1:9) (C). 1mL of blood was placed in to a glass tube with the height and diameter of 7.5cm×10cm respectively [14,15], to measure the CT.

Laboratory evaluation of samples to select healthy dogs

The EDTA blood (Sample A) was used to perform FBC, ALT, AST, Fibrinogen, TP, and Alb tests. These tests were performed to rule out anaemia, thrombocytopenia, and their underlying causes and hepatic disorders. Sample B (with tri-sodium citrate) was centrifuged at ×2000g for 10 minutes at 25°C, and plasma was separated. The semi-automated biochemistry analyser (ERBA Chem-7, Germany) was used to perform PT and aPTT tests.

Determination of Prothrombin time (PT)

The Biolabo method was used for measuring PT. Serum sample of 100µL (from sample B) was pre-warmed at 37°C for 2 minutes in the incubator, and then was placed in the cuvette holder in order to incubate at 37°C for 20 seconds in the semi-automated biochemistry analyser (ERBA Chem-7). Then 200µL of thromboplastin reagent (BIO-TP LI (low ISI) Test kit, France) which has also been pre-warmed at 37°C was added into it and the start key of the analyzer was pressed simultaneously. Next the indicated PT value at the time of formation of the clot was recorded in seconds. The thromboplastin reagent used in the testing was freeze-dried thromboplastin (relatively crude extracts of thromboplastin-rich tissue, from rabbit brain and Ca²⁺).

Determination of activated Partial thromboplastin time (aPTT)

The Helena, Ellagic Acid method was used for measuring aPTT. The semi-automated biochemistry analyser (ERBA Chem-7, Germany) was used and the selected 45 samples were used in this process. Serum sample of 100µL from sample B was pre-warmed at 37°C for 2 minutes in the incubator. It was then transferred to the cuvette holder and 100µL of Ellagic acid solution pre-warmed at 37°C was added and incubated for exactly 3 minutes. The start key of the analyzer was pressed simultaneously; as 100µL of pre-warmed CaCl₂ (0.02 mol/L) at 37°C was added. Then the reading of aPTT value was recorded in seconds.

Determination of CT

BSAVA and WHO guideline [14,15] were followed in this procedure. Blood collected in the glass tube (the height and diameter of 7.5cm×10cm) (C) was rotated well to achieve good surface contact and the tube was immediately placed in an incubator at 37°C until a visible clot was formed while the tube was gently tilted every 30 seconds in order to inspect

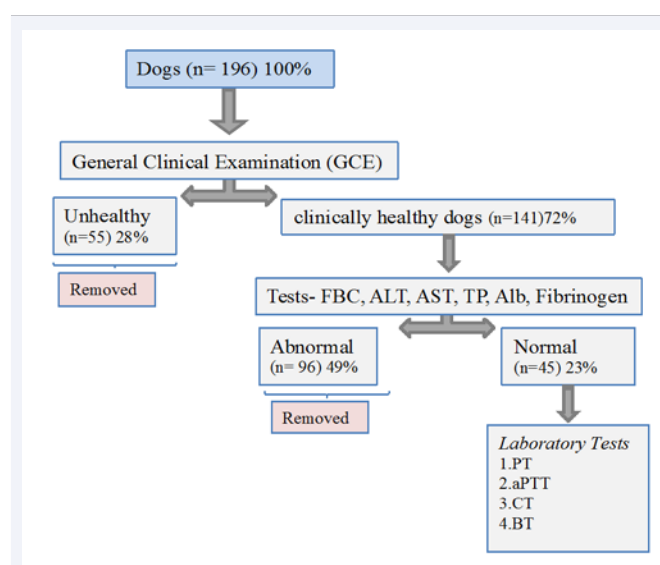


Figure 1 Selection method of dogs for measuring Prothrombin Time (PT), activated Partial Thromboplastin Time (aPTT), Clotting Time (CT), and Bleeding Time (BT).

formation of the clot. The time taken to form a visible clot was then recorded as CT. In order to perform coagulation testing, the general recommendation is to centrifuge and remove the plasma within 1 hour and test it within 4 hours of blood collection [6,16].

etermination of BT

The test procedure for the BT was adopted with reference to the techniques described in Thrall et al., 2012 [17]. The inner surface of the pinna with appropriate thickness of the selected ear was clipped and cleaned with 70% alcohol pad, taking care not to rub the area, and was allowed to dry. Sterile lancet was used to cut 1mm depth area in the aseptically prepared ear lobe.

A timer was started simultaneously. Bleeding from the cut surface was allowed for 30 seconds. The first drop of blood was absorbed on the edge of a circular filter paper without touching the cut surface of the skin. Absorbing the drop of blood from the wound edge was done in every 30 seconds until the bleeding stopped and the time was recorded. The time interval between the cut and cessation of bleeding was recorded as BT.

Statistical analysis

Using MINITAB 16 software package, raw data were checked for normality. Thereafter, Anderson Darling test was performed in order to calculate confidence limits for parameters for each test. The reference ranges for PT, aPTT, CT and BT was calculated. The 95% confidence interval for the lower and upper limits were obtained using Least Significant Difference [18].

RESULTS

In order to obtain healthy individuals, 196 dogs were subjected to collect samples. However, there were only 45 individuals were left as healthy dogs. Out of them the majority (n=29) were females. The represented the large sized breeds (Rottweiler, Labrador Retriever, golden Retriever, Doberman, German Shepherd, Boxer and Ridge Back) and medium sized breeds (Cross-bred and Mongrel). The average age of an individual which was subjected to study was 2.8 years ((Range 0.6-7.0 years).

Data distribution obtained for each test are depicted in Figure 2. The descriptive statistics of PT, aPTT, CT and BT are given in Table 1 and the data were satisfactorily normally distributed. In accordance to the Anderson-Darling test, PT, aPTT, and CT and BT data of selected samples were normally distributed ($P>0.05$).

DISCUSSION

The reference values for coagulation tests, including PT, aPTT, CT, and BT for dogs in Sri Lanka have been based on the studies carried out in Western countries so far. Thus the reference ranges used for PT, aPTT, CT and BT were (6-7) seconds, (9-11) seconds, (3-13) minutes and (1-5) minutes, respectively. However, reference values for PT, aPTT, CT, and BT obtained in this study were (7-11) seconds, (11-22) seconds, (3-12.5) minutes and (0.5-5) minutes respectively. 139 healthy fasting purebred dogs in France determined canine reference intervals for (PT), (aPTT), fibrinogen and antithrombin (AT)

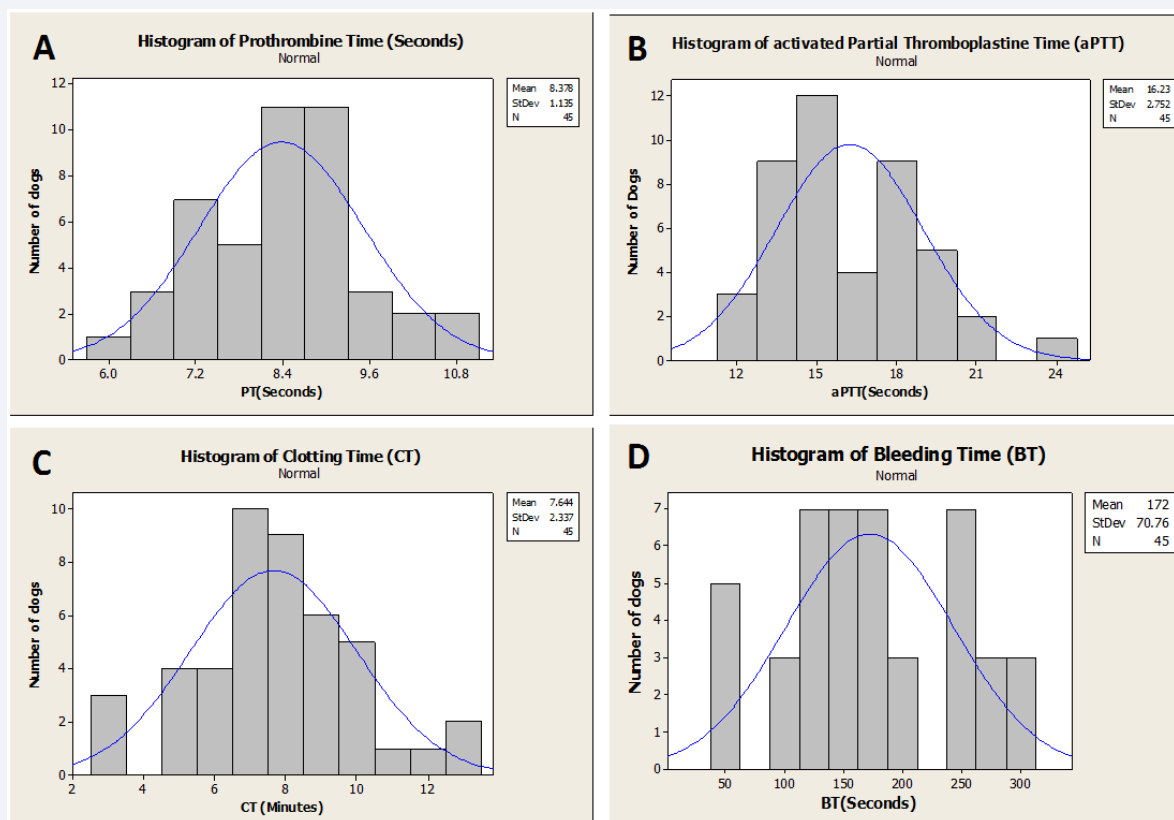


Figure 2 Normal distribution of data. A). Prothrombin Time (PT), B). Activated Partial Thromboplastin Time (aPTT), C). Clotting Time (CT), D). Bleeding Time (BT).

Table 1: Descriptive statistical values obtained for selected tests of PT, APTT, CT, and BT.

Character	P-Value	Mean	2SD	95% confidence interval		Range
				Lower limits	Upper limits	
PT	0.390	8.378	2.27	5.5 - 6.7 s	10.0 - 11.2 s	7-11 s
aPTT	0.116	16.230	5.504	6.29 - 15.16 s	17.3 - 26.17 s	11-22 s
CT	0.282	7.644	4.674	1.32 - 7.23 s	9.11 - 16.64 s	3-12.5 min
BT	0.087	172	141.52	18.46 - 149 s	229 - 394 s	29-308 s

SD-Standard Deviation, s-seconds, min- minute

according to international recommendations which were 6.9-8.8 seconds and 13.1-17.2 seconds, respectively [19]. It has also been reported that significant differences in coagulation test results could be due to the use of different reagents, especially for aPTT, has reported [20]. Slightly lower values for reference interval in dogs than the results of [19] i.e. for PT and aPTT in dogs were 5.7- 8.0 seconds and 10.0- 14.3 seconds, respectively. Therefore, it is important to establish a reference interval in dogs for each laboratory suitable for the specific conditions for that particular laboratory [19]. The aPTT of dogs can be prolonged with deficiencies (<30%) of any one of factors VIII (including Von Willebrand's factor), IX, X, XI or XII, a deficiency of fibrinogen, factor I (<0.5g/l), and/or due to an anticoagulant. The PT is prolonged, if there is a deficiency of factor VII or X (<30%), factor II (prothrombin) or factor I (fibrinogen, <0.5g/l). It is prolonged with severe liver disease, disseminated intravascular coagulation (DIC) or vitamin K deficiency [9]. The reference ranges reported in this report are different to published values with regard to PT and aPTT. However, the reference values reported here with regard to CT and BT are in agreement with similar reference intervals in western countries. In relation to the selected method of samples for this study, the results can be applied to the normal dog population in the country; in fact, expected test values of coagulation parameters will vary depending on the technique used, the method of clot detection, temperature, pH, collection technique, type of anticoagulant and storage condition and time [21]. Studies conducted by Geffre [19], also indicated that significant differences in coagulation test results could be due to different reagents used. Snake envenomation is a condition which requires emergency veterinary assistance as it can lead to haemorrhage due to coagulation disorders, systemic organ damage and death. Minimum laboratory information required to evaluate a patient with a haemostatic defect are, PT, aPTT, CT, BT, and the platelet count. Coagulation tests are essential in the initial diagnostic procedures as well as in the subsequent monitoring of a patient with snake envenomation. Changes of coagulation tests are also one of the diagnostic tools to decide the venom concentration and the identification of the type of snake involved [22]. The basic coagulation panel is also helpful to assess the extent of snake bite envenomation in dogs. Since snake envenomation is a frequent occurrence throughout the year in dogs in most parts of Sri Lanka, it is essential to have appropriate diagnostic and treatment protocols established.

CONCLUSION

This study established standard reference intervals for coagulation tests of PT, aPTT, CT, and BT for dogs. The result

of this study would undoubtedly help when treating dogs with coagulation disorders.

DECLARATIONS

Ethics approval and consent to participate

All experimental procedures and animal care had been approved by the Faculty Ethics Committee (Ref No.VER-14-012), Faculty of Veterinary Medicine and Animal Science, University of Peradeniya, Sri Lanka.

Consent for publication

The authors declare that they have no competing interests.

Availability of data and materials

The dataset(s) supporting the conclusions of this article is included within the article.

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