UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

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PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS VETERINÁRIAS

AVALIAÇÃO DA HEMOSTASIA EM CÃES: FATOR DE VON WILLEBRAND E TEMPO DE PROTROMBINA E TROMBOPLASTINA PARCIAL ATIVADA

MAGNUS LARRUSCAIM DALMOLIN

PORTO ALEGRE

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RESUMO

A doença de von Willebrand (DvW) é um defeito qualitativo/quantitativo do fator de von Willebrand (FvW), uma glicoproteína que desempenha um papel essencial na adesão e agregação plaquetária. Cães acometidos por esta diátese hemorrágica hereditária podem ser assintomáticos, ou apresentar sinais clínicos de um problema hemostático primário, especialmente hemorragias em superfícies mucosas. O diagnóstico da DvW baseia-se na quantificação do FvW plasmático, este sendo atualmente realizado por ELISA – antígeno FvW (Ag:FvW). O presente trabalho desenvolveu um ensaio para quantificação do Ag:FvW em amostras caninas e aplicou o teste em uma população de cães. Também determinou intervalos de referência para o ensaio e para tempos de coagulação. O ensaio apresentou R^2 médio de 0,9810, com coeficientes de variação intra-teste de 1,83 a 4,54% e entre-teste de 9,02 a 17,75%. Valores de referência para Ag:FvW, Tempo de Protrombina e Tempo de Tromboplastina Parcial Ativada obtidos foram de 24,87 a 224,5%, 6,0 a 9,3 segundos e 15,2 a 24,5 segundos, respectivamente. Completando, foi feita uma revisão da literatura sobre a doença em cães. Em conclusão, a determinação do Ag:FvW é um teste essencial para pacientes com histórico de diátese hemorrágica sem coagulopatia e/ou trombocitopenia e pode ser conclusivo para o diagnóstico de DvW. Obter valores de referência para a população local e padronizar os reagentes e instrumentos utilizados são extremamente importantes para um diagnóstico acurado dos distúrbios de hemostasia. Finalmente, um caso clínico de um cão com DvW também foi descrito.

Palavras chave: hemostasia, coagulação, ELISA, valores de referência.

HEMOSTASIS EVALUATION IN DOGS: VON WILLEBRAND FACTOR, PROTHROMBIN AND ACTIVATED PARTIAL THROMBOPLASTIN TIME

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ABSTRACT

The von Willebrand disease (vWD) is a von Willebrand factor (vWF) quantitative/qualitative defect. This glycoprotein plays a crucial role on platelet adhesion and aggregation. Dogs affected by the hemorrhagic diathesis may be asymptomatics, or show clinical signs of a primary hemostatic disturbance, such as bleeding from mucosal surfaces. The diagnosis is based on vWF quantification by ELISA techniques – vWF antigen (vWF:Ag). The current study developed an assay for vWF:Ag quantification on canine samples, and the test was conducted on a dog population. Also, reference intervals were determined for this assay and for clotting times. The assay achieved a mean R^2 of 0.9810, with coefficient of variation for intra-assay of 1.83 to 4.54% and for inter-assay of 9.02 to 17.75%. The vWF:Ag assay, Prothrombin Time and activated Partial Thromboplastin Time reference intervals were 24.87 to 224.5%, 6.0 to 9.3 seconds and 15.2 to 24.5 seconds, respectively. In addition, a literature review about the disease in dogs was done. In conclusion, vWF:Ag determination is an essential assay for patients with hemorrhagic diathesis history without coagulopathy and/or thrombocytopenia, and might be conclusive for the disease diagnosis. Reference values for local population and standard protocols are extremely important for an accurate diagnosis of disorders of hemostasis. Finally, a case report of a dog with vWD was also described.

Key words: hemostasis, coagulation, ELISA, reference values.

SUMÁRIO

1 INTRODUÇÃO

A doença de von Willebrand (DvW) é a desordem hemorrágica hereditária mais comum em cães e já foi diagnosticada em mais de 50 raças (JOHNSON et al., 1988). É particularmente comum no Dobermann, Terrier Escocês e Pastor de Shetland, e é transmitida como uma característica autossômica (JOHNSTONE; CRANE, 1981; RAYMOND et al., 1990; BROOKS et al., 1992; STOKOL et al., 1995b; STOKOL et al., 1995c; RIEHL et al., 2000). A DvW resulta de uma deficiência quantitativa e/ou qualitativa do fator de von Willebrand (FvW), uma glicoproteína plasmática multimérica que atua na adesão das plaquetas ao colágeno subendotelial durante a hemostasia primária. Os multímeros do FvW com elevada massa molecular apresentam maior função hemostática. É classificada como um defeito extrínseco à plaqueta, em que a falha da resposta hemostática é atribuída à deficiência de um fator plasmático necessário para a função plaquetária normal, e não um defeito primário da plaqueta (JOHNSTONE, 2002a; SMITH, 2010). A deficiência é classificada em 3 categorias (conforme a classificação em humanos): DvW tipo 1 (deficiência parcial do FvW), tipo 2 (deficiência qualitativa do FvW) e tipo 3 (ausência do FvW), sendo o tipo 1 a mais comum (BROOKS; CATALFAMO, 2010).

A manifestação clínica da DvW é variável e depende de diversos fatores. A extensão da deficiência e o tipo de DvW são as variáveis mais importantes para determinar a probabilidade e a severidade da hemorragia. Como regra geral, quanto menor a concentração de FvW maior a probabilidade de hemorragia. Uma deficiência absoluta do FvW (DvW tipo 3) resulta em uma hemorragia severa, e necessita de terapia de reposição do fator. Reduções menos dramáticas ocorrem na DvW tipo 1, e consequentemente apresentam manifestações mais leves (STOKOL, 2012). A maioria dos pacientes com a forma leve apresenta sinais clínicos que se manifestam em traumas, troca de dentes decíduos ou hematúria. Pode ocorrer hemorragia intra-operatória ou pós-operatória com risco de morte em alguns casos (BROOKS; CATALFAMO, 2010). Cães com o tipo 2 da doença apresentam deficiência dos multímeros do FvW de elevada massa molecular, e consequentemente apresentam hemorragias severas, apesar da concentração total

do antígeno do FvW (Ag:FvW) ser normal ou discretamente diminuída (STOKOL, 2012).

Existem vários testes para o diagnóstico de DvW, que incluem testes genéricos de hemostasia primária (contagem de plaquetas e tempo de sangramento da mucosa bucal) e testes confirmatórios (concentração do Ag:FvW ou análise genética). A concentração do FvW é medida em ensaio imunológicos com o uso de anticorpos, tipicamente através de ELISA, que detectam epítopos na proteína - Ag:FvW (SLAPPENDEL et al., 1992). Animais com concentrações de Ag:FvW abaixo de 50% são considerados com DvW; aqueles que apresentam concentrações do Ag:FvW superior a 70% são considerados normais. Pacientes com resultados entre estes dois valores são considerados "indeterminados" ou "suspeitos". É importante que o teste seja padronizado para a detecção do Ag:FvW em plasma canino, pois os testes não são produzidos da mesma forma (BENSON et al., 1992). A determinação do Ag:FvW é indicada para o diagnóstico de DvW em um paciente com hemorragia, enquanto os testes genéticos (quando disponíveis) é superior para a detecção de cães portadores (STOKOL, 2012).

O gene do FvW canino está localizado no cromossomo 27, em uma região que é sintênica ao cromossomo humano 12p13.2. O DNAc do gene do FvW canino é composto por 8601 pares de bases, e prediz uma sequência de 2813 aminoácidos (http://www.ncbi.nlm.nih.gov/nuccore/AF099154.1). Embora a estrutura integral de éxons e íntrons do gene canino não foi determinada, alguns dados de splicing em íntrons sugerem que as estruturas dos genes canino e humano sejam muito similares (VENTA, 2000). Vários dos epítopos do FvW são conservados nas diferentes espécies de mamíferos, o uso de anticorpos que apresentam reações cruzadas tem sido amplamente utilizado para a determinação do Ag:FvW (BENSON et al., 1992). Como os FvW humano e canino possuem 86,2% de identidade, uso de anticorpos anti-FvW humano que reagem contra o FvW canino pode permitir o diagnóstico da DvW, assim como também a diferenciação entre os tipos 1 e 3 da doença (BENSON et al., 1991; JOHNSTONE; CRANE, 1991). O presente trabalho abordou uma revisão sobre a doença e desenvolveu um ELISA para a quantificação do FvW em plasma canino, utilizando anticorpos (IgG de coelho purificada de soro e anticorpos monoclonais)

anti-FvW humano. Este trabalho também estabeleceu valores de referência para Tempo de Protrombina e Tempo de Tromboplastina Parcial Ativada em uma população de cães de Porto Alegre. Além disso, um caso clínico de doença de von Willebrand em um cão foi descrito.

2 OBJETIVOS

- Desenvolver e padronizar um ELISA para quantificação de FvW em cães.
- Quantificar o Ag:FvW canino em pacientes clinicamente saudáveis e em pacientes com distúrbio de hemostasia.
- Quantificar o Ag:FvW canino em diferentes raças de cães.
- Correlacionar a quantificação do Ag:FvW canino com a atividade coagulante do fator VIII.
- Determinar um intervalo de referência para Tempo de Protrombina (TP) e Tempo de Tromboplastina Parcial Ativada (TTPA) em uma população de cães de Porto alegre.
- Descrever um caso clínico de doença de von Willebrand tipo 1 em um cão.

3 RESULTADOS

Os resultados serão apresentados em formato de artigo científico e serão submetidos para publicação em periódico da área.

3.1 Artigo de Revisão

CAUSE, DIAGNOSIS AND TREATMENT OF CANINE VON WILLEBRAND DISEASE

Magnus Larruscaim Dalmolin, Mariana Loner Coutinho, Itabajara da Silva Vaz Junior

Abstract

Canine von Willebrand disease is a quantitative/qualitative defect of the von Willebrand factor, a large protein that plays a critical role in platelet adhesion and aggregation. Dogs affected by this hereditary bleeding diathesis may present no clinical evidence of a bleeding problem, or show clinical signs of a primary hemostatic problem, especially hemorrhage from mucosal surfaces. Here we summarize etiology, classification, clinical signs and the recommended diagnostic procedures for canine von Willebrand disease. Treatment options and adequate transfusion support are also covered.

Key-words: von Willebrand disease; von Willebrand factor; dogs; inherited bleeding disorder; hemostasis; transfusion therapy.

Introduction

In 1926, Erik Adolf von Willebrand described a human condition in Finland characterized as an inherited hemorrhagic diathesis. This description included gum bleeding after tooth extraction and excessive hemorrhage from trivial wounds and from the female genital tract (VON WILLEBRAND, 1926). However, joint bleedings, common in hemophilia, were relatively rare. Von Willebrand concluded that the condition was a previously unknown form of hemophilia affecting both sexes, and he called the disease hereditary pseudohemophilia (VON WILLEBRAND, 1931). The prolonged bleeding time was its most important feature. In the 1950s, after the development of methods to determine clotting factors, the condition was named von Willebrand disease (NILSSON et al., 1957; HOLMBERG; NILSSON, 1975).

In 1970, a hemorrhagic diathesis analogous to the human von Willebrand disease (vWD) was reported in a family of German shepherd dogs. The disease profile was characterized by prolonged bleeding time, factor VIII reduced activity, abnormal platelet adhesiveness, marked increase in factor VIII levels after transfusion with normal or hemophilic plasma, autosomal inheritance with variable penetrance, and mild to severe bleeding diathesis (DODDS, 1970). The same author reported additional characteristics of the disease in three successive generations of affected dogs from the original family. These characteristics included a disease progressively less severe with advancing age and repeated pregnancies, reduced ristocetin-induced platelet aggregation, and markedly delayed hemostatic plug formation (DODDS, 1975).

The condition consists of a quantitative/qualitative deficiency of vWF, a large plasmatic glycoprotein necessary for platelet adhesion to vascular damage sites (SADLER, 2005a). As such, it is classified as an extrinsic platelet defect that involves failure in platelet response due to the deficiency of a plasma factor necessary for normal platelet function, rather than a primary defect in the platelet itself (JOHNSTONE, 2002a).

Hemostasis

The hemostasis consists in the equilibrium between bleeding and thrombosis. If one of these occurs, the organism must have mechanism to stop, and reestablish the normal blood flow (SMITH, 2010). Primary hemostasis consists of a vascular phase and a platelet phase. Following the severing of vessels, a reflex vasoconstriction temporarily retards blood flow, allowing time for formation of the platelet plug to begin and coagulation to commence. The damage or removal of endothelial cells exposes the subcellular matrix, resulting in platelet adhesion. The adhesion process is mediated through von Willebrand factor, which allows the subendothelial collagen and platelet interaction. After the platelet adhesion, there is ADP (adenosine diphosphate) release; witch elicits platelet aggregation in the presence of Ca^{2+} ions. Following vessel injury, tissue factor (TF) on subendothelial adventitial cells activates coagulation. In addition, platelets provide membrane phospholipids and membrane surface for the coagulation cascade (HARVEY, 2012).

Secondary hemostasis is composed of coagulation and consolidation of the temporary hemostatic platelet plug into a definitive hemostatic plug. Coagulation is an enzymatic process involving the conversion of proenzymes to active enzymes. Some activated coagulation factors are themselves enzymes, and others (factors V and VIII) are cofactors that combine in complexes to generate specific enzymatic activities. A series of enzymatic reactions, each producing active enzymes, results in an amplification of the original stimulus to initiate coagulation. Ca^{2+} ions are required for multiple reactions in coagulation. The final product of coagulation is the formation of cross-linked fibrin strands around and, to a lesser extent, through the platelet plug, making it stronger and decreasing the likelihood that rebleeding will occur (HARVEY, 2012).

Pathogenesis

Megakaryocytes and endothelial cells make von Willebrand factor (vWF) from a large precursor that dimerizes in the endoplasmic reticulum through C-terminal cysteine knot domains (SADLER, 1994). vWF is stored in secretory granules, the Weibel-Palade body in endothelial cells, and the α-granule in platelets. The secreted multimers can be >20,000 kDa in mass and 4 μm in length (FOWLER et al., 1985). vWF acts as a carrier for coagulation factor VIII (FVIII), circulating with FVIII in a noncovalent complex. Upon vascular injury, vWF binds to subendothelial collagen and undergoes a conformation change that facilitates its interaction with platelet glycoprotein Ib (Figure 1). vWF also mediates intraplatelet bridging via platelet glycoprotein IIb/IIIa. High molecular weight (MW) vWF multimers are most effective in supporting platelet adhesion. Because of its interaction with FVIII, in some patients the vWD is associated with a circulating FVIII deficiency (STOKOL et al., 1995a; SMITH, 2010).

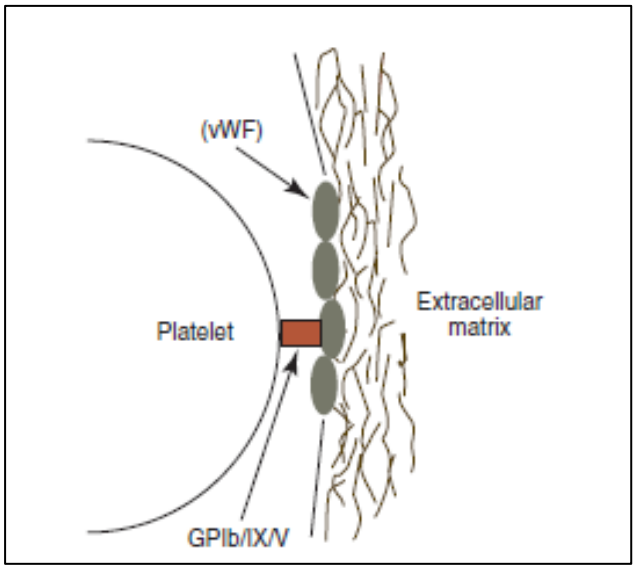

Figure 1. von Willebrand factor (vWF) is needed for optimal adhesion of platelets to the subendothelial matrix under flow conditions. vWF binds to a glycoprotein complex GPIb/IX/V receptor on the platelet surface (HARVEY, 2012).

vWD is the most common inherited hemorrhagic disease in dogs, affecting more than 50 breeds. It is particularly common in Doberman, Scottish terrier and Shetland sheepdog breeds, and is transmitted as an autosomal trait (JOHNSTONE; CRANE, 1981; RAYMOND et al., 1990; BROOKS et al., 1992; STOKOL et al., 1995b; STOKOL et al., 1995c; RIEHL et al., 2000). A prevalence of von Willebrand factor deficiency of 1.43% was described in a population of dogs without clinical evidence of hemorrhage (MATTOSO et al., 2010). However, the real prevalence of vWD within affected breeds is difficult to determine. In the absence of random surveys, diagnose bias favors screening affected individual and their relatives (BROOKS et al., 2001).

Inherited vWD

vWD is classified into three types based in the pathophysiology of the disease (table 1). Type 1 vWD is a partial quantitative deficiency, with low

plasma vWF concentration (less than 50% of normal), although the protein consists of a full complement of functional multimers. This type of vWD is the most common form of the disease in dogs, with cases in several pure- and mixed-breed dogs (JOHNSTONE, 2002a; BROOKS; CATALFAMO, 2010; STOKOL, 2012). Homozygous and most heterozygous carriers of type 1 vWD have plasma vWF antigen (vWF:Ag) concentrations of less than 50%, with a normal multimer distribution profile. However, in some heterozygotes vWF:Ag concentrations are in the low-to-normal range (50-75%), making it difficult to differentiate these carriers from unaffected dogs. Causative mutations for type 1 vWD are found throughout the vWF gene and in some variants, mutations unlinked to the vWF gene locus influence vWF protein stability or rate of synthesis (RIEGER et al., 1998; RIEHL et al., 2000; BROOKS; CATALFAMO, 2010). Type 1 disease is often a mild disorder, tending to be troublesome rather than life-threatening, particularly in dogs that have undergone surgery or suffered a traumatic injury (JOHNSTONE, 2002a).

Type 2 vWD describes a group of vWF functional variants. Although four distinct subtypes are found in humans (table 1), type 2A vWD is the only subtype described in dogs. Type 2 includes patients with qualitative changes in multimers and lower numbers of large multimers. The residual vWF's ability to support platelet adhesion is greatly reduced (BROOKS; CATALFAMO, 2010). Type 2 vWD represents 10-20% of human cases (SADLER, 2005a). However, this type was described only in two dogs. In general, type 2 vWD mutations are clustered in vWF gene regions required for multimer assembly and stability, or vWF-platelet interactions breeds (VAN DONGEN et al., 2001; KRAMER et al., 2004; GAVAZZA et al., 2012). Type 2 vWD is usually a severe clinical entity (JOHNSTONE, 2002a).

Table 1 - The human von Willebrand disease classification.

Type	vWF defect
1	Partial quantitative defect, vWF with normal structure and function
2A	Selective loss of high MW vWF multimers, decreased platelet-vWF and collagen interaction
2B	Increased affinity of vWF to platelet glycoprotein Ib
2M	Impaired vWF binding to platelet glycoprotein Ib, normal multimer structure
2N	Decreased vWF binding to FVIII
3	Complete vWF deficiency

Adapted from BROOKS AND CATALFAMO, 2010.

In its homozygous expression, type 3 vWD is invariably a clinically severe hemostatic disorder, and bleeding episodes can be life-threatening. Homozygous individuals usually have little or no detectable plasma vWF:Ag (<1% of normal). Heterozygotes have subnormal to low-normal plasma vWF:Ag concentrations, and are frequently asymptomatic. In many species, the lack of vWF is associated to lower plasma FVIII levels. In comparison to human, dogs with vWD type 3 have mild clotting factor reduction due to higher FVIII activity on this specie (STOKOL et al., 1995a; JOHNSTONE, 2002a; BROOKS; CATALFAMO, 2010). This form was described as familiar trait among several breeds (Dutch Kooiker, Scottish Terrier, Shetland sheepdog), and there are sporadic reports in Border Collies, Cocker Spaniels, Labrador Retrievers, Malteses, Pit Bulls, and mixed breeds. Common mutations in type 3 patients include frameshift, nonsense, and large deletions that disrupt vWF synthesis (JOHNSTONE et al., 1993; SLAPPENDEL et al., 1998; VENTA et al., 2000; PATHAK, 2004; VAN OOST et al., 2004). Known canine vWD mutations are summarized in table 2.

Table 2 - Canine von Willebrand disease mutations.

Breed	vWD Type	Mutation
Doberman[a]	Type 1	Exon 43, codon TCG to TCA, enhancement of cryptic splice site (premature stop)
German Shorthair Pointer	Type 2	Exon 28, codon AAT to AGT (N[c] to S[d])
German Wirehair Pointer	Type 2	Exon 28, codon AAT to AGT (N[c] to S[d])
Scottish Terrier	Type 3	Exon 4, 1-bp[b] deletion, frame shift (premature stop)
Dutch Kooiker	Type 3	Intron 16, splice site GT to GA, alternative splicing, (premature stop)
Shetland Sheepdog	Type 3	Exon 7, 1-bp[b] deletion, frame shift (premature stop)

[a]Bernese Mountain Dog, Coton de Tulear, Drentsche Patrijshond, German Pinscher, Kerry Blue Terrier, Papillion, Pembroke Welsh Corgi, Poodle e Stabyhoun have the same mutation as Doberman Pinschers, [b]base pair, [c]asparagine, [d]serine. Adapted from BOUDREAUX, 2012.

Acquired vWD

Acquired vWD (AvWD) refers to quantitative/qualitative defects of vWF as consequence of other pathologies. Antibody-mediated clearance secondary to an autoimmune disease, shear-induced proteolysis due to cardiac disease, and increased vWF-platelet binding in thrombotic and neoplastic syndromes have been reported in humans (VELIK-SALCHNER et al., 2008; COUCKE et al., 2014; LEE et al., 2014). Also, AvWD has been described in humans in association with hypothyroidism and treatment with plasma expanders (DE et al., 2001; STUIJVER et al., 2014). In addition, a loss of high MW vWF multimers

has been recognized in dogs with turbulent flow due to lesions in cardiac valves (TARNOW et al., 2004; TARNOW et al., 2005).

Clinical Manifestations

vWD manifests with clinical signs typical of a primary hemostatic disorder, with excessive bleeding from mucosal surfaces that are rich in fibrinolysins, including gum hemorrhage, epistaxis, melena, hematuria and uterine hemorrhage. Excessive hemorrhage after surgery or trauma (including clipping of claws) is often the first sign in dogs (DODDS, 1970; DODDS, 1975; JOHNSTONE; CRANE, 1981; BROOKS et al., 1992; BROOKS et al., 2001). Petechiae are observed infrequently in dogs with vWD (in contrast to dogs with thrombocytopenia), which may be a useful diagnostic clue when trying to differentiate clinically between primary hemostatic disorders. Other signs that have been observed in vWD include lameness, intracranial hemorrhage and poor wound healing (STOKOL, 2012). In an epidemiologic study with 260 dogs with vWD, Brooks et al. (1992) found a predominance of bleedings from mucosal surfaces and cutaneous lesions after trauma or surgery. Mucosal bleeding was particularly common in Scottish Terriers and Shetland sheepdogs, while Doberman dogs were predisposed to urogenital bleedings.

The clinical expression of vWD is variable, with many affected dogs not presenting any evidence of excessive bleeding. Excessive bleeding is difficult to document and quite subjective. There is an association between the clinical expression and the concentration of vWF or type of vWD, and the likelihood of hemorrhage is higher in lower vWF concentrations. In contrast, the genetic defect or status (heterozygous or homozygous) has not been linked conclusively to disease expression. The critical threshold of vWF:Ag (the concentration below which a dog is considered “at risk” of hemorrhage) varies from 25-40% in dog with type 1 vWD, although not necessarily a dog with concentrations below this threshold will bleed excessively (STOKOL, 2012). Doberman Pinschers with vWD type 1 marker locus and low vWF:Ag concentrations may not have abnormal bleeding. This indicates that the expression pattern appears to be more complex than that of a simple recessive trait (BROOKS et al., 2001).

Some dogs may hemorrhage despite vWF concentrations above 40%. It is arguable whether the hemorrhage can be truly attributed to vWD and, in many cases, affected animals have concurrent thrombocytopenia, an underlying disease, or undergo drug therapy that may affect platelet function. Type 2 and type 3 vWD causes severe hemorrhage in affected dogs. Considering that acquired diseases or drug may precipitate or worsen hemorrhage in any dog with vWD, bleeding does not always manifest at a young age (STOKOL, 2012).

Diagnostic Tests

Specific assays of canine vWF are needed to diagnose vWD in dogs, as most dogs with vWD have normal platelet counts and coagulation profile - prothrombin time (PT), activated partial thromboplastin time (APTT), and fibrinogen level. However, some very severely deficient dogs have low factor VIII clotting (FVIII:C) activity, only enough to show a slightly prolonged APTT. So, these tests should not be used as confirmatory diagnostic test for vWD (BARR; MCMICHAEL, 2012). Numerous vWD diagnostic tests have been developed, including screening tests such as bleeding time, and specific tests, like the vWF:Ag assay and some genetic tests. The vWF multimeric analysis is commonly used as a research tool and to diagnose subtypes of vWD type 2, but it has been recently replaced by the collagen-binding assay (FAVALORO, 2007; STOKOL, 2012).

Dogs with excessive hemorrhage attributed to a primary hemostatic disorder, normal platelet counts and coagulation panel should be submitted to vWD screening or the vWF:Ag assay. The screening tests include the buccal mucosa bleeding time (BMBT) and platelet function analysis. When results of the screening test are abnormal, specific tests are required to establish whether bleeding episodes are due to vWD. Since the vWF:Ag concentration correlates with clinical signs, this assay can be performed without screening tests (BURGESS et al., 2009).

vWF:Ag

The current gold standard for the diagnosis of the disease is the vWF:Ag test. The assay determines concentration of the factor as opposed to function (BURGESS; WOOD, 2008). The vWF concentration is determined with antibodies by immunologic assays, typically through ELISA, which detect the vWF as an antigen vWF:Ag (SLAPPENDEL et al., 1992). While the vWF:Ag assay is indicated to diagnose vWD in a dog with bleeding diathesis, genetic tests are the best choice to detect a carrier (BROOKS et al., 1996; VAN OOST et al., 2004; GAVAZZA et al., 2012).

Test results of vWF (and clotting factors) are conventionally reported as percentage on a normal value basis. The concentration of each test sample is compared with that of a reference standard having an assigned value of 100% or 100 units per deciliter (U/dL). Assay standards and reference intervals for each species vWF:Ag have to be established by each testing laboratory. In general, plasma vWF:Ag below 50% (<50 U/dL) indicates vWF deficiency (JOHNSTONE; CRANE, 1991; SADLER, 1994; BROOKS; CATALFAMO, 2010). In type 1 vWD, critical limits of vWF:Ag concentration can be used as approximate rough estimate of hemorrhagic risk or response to transfusion therapy; however, results do not always correlate with clinical signs (BURGESS et al., 2009).

As epitopes of vWF are conserved in different species of mammals (human and canine vWF are 86.2% identical at amino acid sequence), cross-reactions have been consistently used to determine vWF:Ag concentration in animals (BENSON et al., 1992). Anti-human vWF antibodies react with canine vWF, which make them suitable for canine vWD diagnosis and even differentiate types 1 and 3 of the disease (BENSON et al., 1991; JOHNSTONE; CRANE, 1991; NICHOLS et al., 2010). The assay should be standardized for the detection of vWF:Ag in canine samples (BENSON et al., 1992).

Collagen-binding Assay

This assay measures the quantity of vWF bound to collagen in a immunoenzymatic procedure. Collagen has been shown to bind vWF with a

preference for the high MW forms; therefore, the collagen-binding (vWF:CB) assay can be used to assess the relative proportion of large vWF multimers (BROWN; BOSAK, 1986). In human patients with vWD, collagen-binding activity is significantly lower, when compared with control patients (PACZUSKI, 2002). In dogs, the vWF:Ag and vWF:CB assays have strong association, and vWF:CB should be included among the vWF-specific assays used to confirm type 2 vWD. However, the difficulties of standardization of this test makes it scarcely available (SABINO et al., 2006; BURGESS; WOOD, 2008; BURGESS et al., 2009).

Buccal Mucosal Bleeding Time (BMBT)

BMBT is an *in vivo* test of primary hemostasis. Bleeding time is performed on the buccal mucosa using a device that delivers incisions with accurate depth and length. The buccal mucosa is reverted and held gently in place with a gauze strip during the test. Time is measured until bleeding stops completely. This technique may require anesthesia, particularly if the animal is fractious, shakes its head, or licks the incisions. BMBT should be performed in patients with normal platelet counts. It can be used as a screening test for vWD (especially on vWD suspected patients, prior to surgery), but it is not sensitive or specific for this disorder, because patients with platelet diseases may also have prolonged bleeding times (CHRISTOPHERSON et al., 2012). In addition, dogs with vWF:Ag concentration below 50% may not have prolonged bleeding times, and therefore normal hemostasis should not be presumed when a dog presents normal BMBT. So, the BMBT seems to be more suitable as a screening test for those patients with normal platelet counts and coagulation profile, with clinical evidence or history of bleeding diathesis due to a primary hemostasis defect (SATO et al., 2000).

BMBT should be used cautiously as a screening test (pre-surgical or otherwise) in dogs with suspected type 2 or 3 vWD. Affected dogs may have infinite BMBT, may suffer from rebleeding, and it may be difficult to stop the induced hemorrhage without replacement therapy or local treatment (STOKOL, 2012).

Platelet Function Analyzer

Global platelet adhesion and aggregation can be analyzed by aperture closure instruments; however, as a diagnostic method, it is not specific to any disorder. The instrument aspirates whole blood through a capillary tube and a compartment containing test cartridge membrane with a central aperture and coated with collagen, and either adenosine diphosphate (ADP) or epinephrine. As blood flows under high shear across the membrane and through the aperture, platelets become activated and begin to adhere and aggregate, primarily via vWF interactions with GPIb and GPIIb/IIIa, resulting in occlusion of the aperture within 3 minutes, under normal physiological conditions - closure time. Closure time (CT) is affected by many variables in addition to platelet function including platelet count, packed cell volume (PCV), vWF concentration, and improper sample handling including errors in anticoagulant to blood ratio (HARRISON, 2005; COUTO et al., 2006; CHRISTOPHERSON et al., 2012). When used associated with the vWF:CB assay and vWF:Ag, this method has proved valuable to identify canine vWD (MISCHKE; KEIDEL, 2003; TARNOW et al., 2005; BURGESS et al., 2009).

Genetic Detection

The canine vWF gene has been localized to canine chromosome 27 (NICHOLS et al., 2010). In several breeds of dogs, von Willebrand disease type I is caused by a G to A transversion of the last nucleotide of vWF exon 43. The mutation activates a cryptic splice site a few nucleotides upstream of the normal splice site, leading to a frame shift that results in the formation of a truncated protein of 119 amino acids (VENTA et al., 2000; VENTA et al., 2004). However, some dogs with this mutation may not have abnormal bleedings (BROOKS et al., 2001). While low plasma vWF concentration is a primary risk factor for expression of type 1 vWD, molecular genetics and biochemical analyses have not fully defined additional factors that influence disease penetrance (BROOKS et al., 2001). In addition, in some variants, described in mice and people, mutations causative for type 1 vWD are found unlinked to the vWF locus, and influence

vWF protein stability or rate of synthesis (BROWN et al., 2003; LEMMERHIRT et al., 2007). Identification of canine vWF genetic mutation is commercially available for specific mutations and breeds, by polymerases chain reaction methods.

For assisting dog breeders with optimal breeding strategies, detection of carriers is best performed using genetic analysis for a specific mutation, if a mutation has already been identified for that specific breed. If the genetic mutation has not been identified or no other genetic tools are available, carrier detection will have to rely on vWF:Ag concentrations. This is important, because there is an overlap in vWF:Ag concentrations between carrier and affected dogs. Genetic analyses should not be used as the sole diagnostic test for vWD in a bleeding animal, since genetic status cannot be used to predict vWF:Ag concentrations, and there is no evidence linking genetic status to excessive bleeding (STOKOL, 2012).

Treatment

Treatment for vWD is palliative, with the goal being short-term prevention or control of hemorrhage. This is best achieved by infusion of plasma products that contain high concentrations of vWF, although desmopressin, a drug that increases plasma vWF:Ag concentration, can also be used. Preventing hemorrhage is usually easier than treating it (prophylaxis is paramount for elective surgical procedures). Drugs that are known to inhibit platelet function, such as non-steroidal anti-inflammatory agents or acetylsalicylic acid, heparin, dextran, and other plasma expanders should be avoided in animals with vWD. Hereditary vWD causes a life-long bleeding tendency. Therefore, invasive procedures should be avoided. In the event of injury or non-elective surgery, local wound care may help reduce transfusion requirements. Cautery, topical tissue adhesive, multilayer closure, and pressure wraps help to reduce blood loss at focal injury sites, but vWF replacement is usually required for treatment of bleeding episodes (BROOKS; CATALFAMO, 2010; STOKOL, 2012).

Cryoprecipitate

The product of choice for prevention or cessation of hemorrhage in vWD is cryoprecipitate, a concentrated form of vWF and FVIII. Cryoprecipitate provides the greatest amount of vWF in a small plasma volume and is associated with few side effects. It is also more effective at increasing plasma vWF:Ag concentrations and shortening BMBT, compared to other blood products (CHING et al., 1994; STOKOL et al., 1997; STOKOL; PARRY, 1998; STOKOL, 2012). Cryoprecipitate also contains fibrinogen, factor XIII and fibronectin. The cryoprecipitation process concentrates to about one-tenth the volume required for an approximately 50% factor activity of the starting fresh frozen plasma (FFP). Volume reduction is the major advantage of cryoprecipitate transfusion; therapeutic levels of the factor are attained within few minutes over the course of a single bolus infusion (BROOKS, 2010). Recommendations for dosing are 1 unit of cryoprecipitate for every 10 kg of body weight. Lyophilized (freeze-dried) canine cryoprecipitate is also an alternative (DAVIDOW, 2013).

Fresh Plasma and Fresh Frozen Plasma

Fresh plasma (FP) and fresh frozen plasma (FFP) are good alternatives to cryoprecipitate, but these methods are associated with more non-hemolytic transfusion reactions, particularly with fast infusion rates. FP/FFP provides maximum quantities of the labile coagulation factor V and VIII and vWF, as well as all other coagulation factors and plasma proteins (GIBSON; ABRAMS-OGG, 2012). FFP is associated with more adverse effects (ranging from mild pruritus to paleness and weakness) than cryoprecipitate in the treatment or prophylaxis of hemorrhagic episodes in dogs with vWD (DAVIDOW, 2013). 15–30 mL/kg of these products are needed to provide adequate factors for dogs with von Willebrand disease (vWD) or hemophilia A (STOKOL; PARRY, 1998).

Fresh whole blood

Fresh whole blood (FWB) contains all blood components (red blood cells, platelets, labile and stable coagulation factors, plasma proteins), and have to be transfused within 8 hours of collection. Unnecessary transfusions of FWB (in non-anemic patients) should be avoided, thus preventing sensitization of the recipient

to foreign antigens and to minimize the risk of future transfusion reactions, because many of these dogs with vWD may suffer recurrent bouts of hemorrhage that eventually will require multiple transfusions. If blood components are always available, the administration of FWB would be restricted to those patients with vWD and concurrent anemia. 12-20 mL/kg of FWB should be transfused if the dog is anemic and hypoxic (GIBSON; ABRAMS-OGG, 2012; STOKOL, 2012).

Non-transfusional therapy

Desmopressin (1-deamino-arginine vasopressin; DDAVP) is a synthetic analogue of arginine vasopressin that has proved suitable as a means to increase vWF:Ag and FVIII in dogs with vWD (SATO; PARRY, 1998; OLSEN et al., 2003). DDAVP increases plasma vWF:Ag concentrations by inducing VWF release from Weibel-Palade bodies in the endothelial cells. The drug is prescribed for the treatment of vWD type 1 in dogs. Clinicians should be aware that repeated injections may reduce response due to storage depletion, so this drug should be used in prophylaxis, rather than in therapy (JOHNSTONE; CRANE, 1987). Prophylactic administration before surgery is recommended for patients with unknown vWD status or those with vWF:Ag concentrations near critical values. However, some patients have low vWF deposits, and may not respond to this drug, and plasma products should be available. A prophylactic injection of 1 µg/kg subcutaneously 30 minutes before the surgery is recommended for dogs with unknown vWD status or those with vWF:Ag concentrations near critical values (CALLAN; GIGER, 2002).

Recombinant human interleukin-11 (rhIL-11), a pleiotropic cytokine that is approved for treatment of thrombocytopenia, has been shown to induce sustainable elevations in vWF and FVIII in dogs (NICHOLS et al., 2000). The mechanism seems to be through up-regulation of vWF mRNA by interleukin-11, instead of vWF release from Weibel-Palade bodies (OLSEN et al., 2001). The administration of rhIL-11 with or without DDAVP may provide an alternative to plasma-derived products for some vWD patients if it is shown safe in clinical trials (OLSEN et al., 2003).

Conclusions

The vWD is the main inherited bleeding diathesis in the dog, affecting several breeds worldwide. Access to diagnostic tools and early detection of affected dogs are central to hemorrhage prophylaxis and treatment. Furthermore, access to blood components, especially plasma products, is of paramount importance for appropriate transfusion support, as these dogs may have bleeding episodes in their lifetime.

Conflict of interest statement

None of the authors of this paper has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

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3.2 Artigo Científico 1

EVALUATION AND VALIDATION OF ANTI-VWF ANTIBODIES TO DETECT VON WILLEBRAND FACTOR DEFICIENCY ON CANINE SAMPLES

Magnus Larruscaim Dalmolin, Eliane Bandinelli, Mariana Loner Coutinho, Itabajara da Silva Vaz Junior

Abstract

Canine von Willebrand disease (vWD) is a von Willebrand factor (vWF) quantitative/qualitative defect. This glycoprotein plays a crucial role on platelet adhesion and aggregation. Dogs affected by the hemorrhagic diathesis may be asymptomatics, or show clinical signs of a primary hemostatic disturbance, such as bleeding from mucosal surfaces. The diagnosis is based on vWF quantification by ELISA techniques – VWF antigen (vWF:Ag). The current study developed an assay for vWF:Ag quantification on canine samples, and the test was conducted on a healthy dog population (n = 220) to determine reference values and to study vWF and factor VIII clotting activity (FVIII:C) behavior. The assay has a mean R^2 of 0.9810, with coefficient of variation for intra-assay of 1.83 to 4.54% and for inter-assay of 9.02 to 17.75%. The reference range for the assay was between 24.87 – 224.5%. A total of 47 (21.36%) dogs with vWF:Ag values below 50% , and 5 dogs (2.27%) with values under the inferior reference range (i.e. 24.87%) were found. Dogs with vWF:Ag <50% have less FVIII:C (P = 0.007). The current assay can be used for canine vWF:Ag determination and even vWD diagnosis, when combined with appropriate clinical evaluation.

Key-words: canine von Willebrand disease, ELISA, dogs, FVIII, monoclonal antibodies.

Introduction

The vWD is the most common inherited hemorrhagic disease in the dog, affecting several breeds and is transmitted as an autosomal trait (JOHNSTONE; CRANE, 1981; RAYMOND et al., 1990; BROOKS et al., 1992; STOKOL et al., 1995b; STOKOL et al., 1995c; RIEHL et al., 2000). The condition consists of a quantitative (types 1 and 3) or qualitative (type 2) deficiency of von Willebrand factor (vWF), a multimeric glycoprotein necessary for the platelet adhesion to vascular damage sites (SADLER, 2005a). The vWF acts as a carrier for coagulation factor VIII (FVIII), circulating with FVIII in a noncovalent complex, and thus protects FVIII from early degradation. Because of its interaction with FVIII, in some patients the vWD is associated with a circulating FVIII deficiency. Upon vascular injury, vWF binds to subendothelial collagen and undergoes a conformation change that facilitates its interaction with platelet glycoprotein Ib. vWF also mediates intraplatelet bridging via platelet glycoprotein IIb/IIIa. High molecular weight (MW) vWF multimers are most effective in supporting platelet adhesion, compared to low molecular weight multimers (STOKOL et al., 1995a; SMITH, 2010).

vWD manifests with clinical signs of excessive bleeding from mucosal surfaces, including gum hemorrhage, epistaxis, melena, hematuria and uterine hemorrhage. Excessive hemorrhage after surgery or trauma (including clipping of claws) is often the first presenting sign in dogs (DODDS, 1970; DODDS, 1975; JOHNSTONE; CRANE, 1981; BROOKS et al., 1992; BROOKS et al., 2001). Other signs that have been observed in vWD include lameness, intracranial hemorrhage and poor wound healing (STOKOL, 2012).

No single test is able to detect all forms of vWD, and the definitive diagnosis of vWD is based on a panel of assays (FAVALORO, 2006). The vWF antigen (vWF:Ag) test is a quantitative enzyme-linked immunosorbent assay (ELISA), that measures the concentration of vWF protein (PATZKE; SCHNEPPENHEIM, 2010). As epitopes of vWF are conserved in different species of mammals, the use of cross-reaction have been widely performed for vWF:Ag concentration in animals (BENSON et al., 1992; SATO; PARRY, 1998). Anti-human vWF antibodies react with canine vWF, which allows canine vWD diagnostic and even the differentiation between types 1 and 3 of the disease

(BENSON et al., 1991; JOHNSTONE; CRANE, 1991; BENSON et al., 1992; NICHOLS et al., 2010). Although this assay is widely recommended for canine vWD diagnosis, it is available at few laboratories in South America, and actually the samples are processed in other countries (USA and Europe). In addition, the need for kit importing and specific sample handling (samples must be transported and stocked frozen) makes the assay expensive and time consuming. The objective of this study is to validate a canine vWF:Ag ELISA using anti-human vWF antibodies. A reference interval for the test is also stablished.

Material and methods

Samples used in this study were obtained with owners consent, and the procedures were approved by the institutional animal care use committee of the Federal University of Rio Grande do Sul (No. 24925).

Healthy canine plasma pool

All dogs were previously submitted to clinical examination (cardiopulmonary auscultation, abdominal palpation and rectal temperature) and laboratory screening (complete blood count, creatinine, albumin and alanine aminotransferase). Blood samples from 24 dogs were collected by atraumatic puncture of jugular vein using a vacuum system. Blood samples were collected into 4.5 mL 3.2% sodium citrate tubes (9 parts blood:1 part citrate) from 12 males and 12 females in range from 1 to 10 year old. The pool comprised Great Danes, Golden Retrievers, Brazilian Bulldogs, Dachshunds, Yorkshires, Boxers and Mongrels. After collection, the citrated blood was centrifuged at 1500 X g for 15 min at room temperature; plasma was harvested, pooled, and then stored in 400 µL aliquots at -80°C until needed. A value of 100% of vWF:Ag was attributed to the canine plasma pool (CPP).

Patient samples

For the vWF:Ag assay standardization, blood from 3 dogs (dog 1 – hemophilia A; dog 2 – type 1 von Willebrand disease; dog 3 – normal dog) were

collected into 4.5 mL 3.2% sodium citrate tubes by atraumatic cephalic, saphenous or jugular venipuncture . Blood samples were centrifuged, plasma was harvested, and stored at -80°C until analyses.

MAbs and rabbit IgG against vWF

Monoclonal antibodies and rabbit IgG to human vWF were provided by Vinícius Sortica, (Laboratório de Hemostasia do Departamento de Genética da Universidade Federal do Rio Grande do Sul).

Briefly, rabbits were immunized with the vWF:Ag purified from lyophilized commercial FVIII/vWF concentrate to obtain serum anti-vWF, and IgG was purified by protein G affinity chromatography. To generate mAbs to vWF, spleen cells of female adult mice BALB/c inoculated with partially purified human vWF were used. The immunoglobulins G produced by hybridoma cells were purified by Sepharose-protein G affinity chromatography (SORTICA et al., submitted).

Antibody/serum reactivity to canine vWF

To determine if the serum and mAbs would react to canine vWF (cvWF), 96-well flat bottom plates (BD Falcon™, BD - NJ, USA) were coated with 1 µg/mL of mAb (vW18 or vW21) or rabbit IgG anti-human vWF on a solution of 0.05 M carbonate-bicarbonate buffer (pH 9.6). The plates were incubated for 16 h at 4°C, and then washed 3 times with a PBS/BSA 0.5% washing buffer. A blocking step with PBS/BSA 0.5% washing buffer was performed for 60 min incubation at 37°C. The blocking solution was removed and samples were added to the plate - CPP, human plasma, cryoprecipitate and cryosupernatant samples. Cryoprecipitate (CRYO) and cryosupernatant were prepared as previously described (STOKOL; PARRY, 1995). The plate was incubated for 60 minutes at 37°C, and another washing step was performed. The mAbs and rabbit IgG peroxidase-conjugated anti human-vWF were added at 1:2000, and the plate was incubated as the previously step. Finally, after the washing step, the reaction was reveled within 15 minutes incubation period (peroxidase substrate, o-phenylenediamine dihydrochloride, Sigma Fast™, Sigma-Aldrich - MO, USA), and a 12.5% solution of sulfuric acid was added to stop the reaction. The plate

was evaluated in spectrophotometer (VersaMax™, Molecular Devices - CA, USA) at 492 nm to determine the best sandwich ELISA.

Checkerboard titration

After we stablish the best antibody combination (see above), the next step was to determine the dilutions of each reagent. A 96-well flat bottom plate was coated with different concentrations (0.5, 1.0, 2.0 and 4.0 µg/mL) of rabbit IgG anti-human von Willebrand factor on a solution of 0.05 M carbonate-bicarbonate buffer (pH 9.6). Samples (CPP, CRYO and cryosupernatant) were added at different dilutions (1:10, 1:20 and 1:40). The mAb vW18 was added at 1:2000. Coating, blocking, washing, and developing of reaction steps were performed as described at the "Antibody reactivity to canine vWF" section.

Then, another ELISA was performed to achieve the vW18 mAb dilution. A 96-well flat bottom plate was coated with 100 ng of rabbit IgG anti-human von Willebrand factor on a solution of 0.05 M carbonate-bicarbonate buffer (pH 9.6). Samples (CPP, CRYO and cryosupernatant) were added at 1:20 dilution. The peroxidase-conjugated mAb vW18 was added at 1:100, 1:500, 1:1000 and 1:2000. Coating, blocking, washing, and revelations steps were performed as described above.

vWF:Ag assay

At coating step, 96-well flat bottom plates were coated with 100 ng of rabbit IgG anti-human vWF on a solution of 0.05 M carbonate-bicarbonate buffer (pH 9.6). The plates were incubated for 16 h at 4°C, and then were washed 3 times with a PBS/BSA 0.5% washing buffer. A blocking step with PBS/BSA 0.5% washing buffer was performed for a 60 min incubation period at 37°C. Then, a standard curve with serial dilutions of a normal canine plasma pool (CPP) representing relative vWF concentrations of 100%, 50%, 25%, 12.5%, 6.25%, 3.12% and 0%, was applied to the plates along with patient samples at a 100% dilution. All the samples were diluted using the PBS/BSA 0.5% washing buffer. After 60 min of incubation period at 37°C, the washing step was repeated and a solution of peroxidase-conjugated mAb at a 1:100 dilution was added and

incubated for 60 min at 37°C. The washing steps was then repeated, and after 15 min incubation period with the peroxidase substrate, o-phenylenediamine dihydrochloride, a 12.5% solution of sulfuric acid was added to stop the reaction. The optical density (OD) was determined in spectrophotometer at 492 nm, and was then plotted against Log vWF:Ag% dilution to form a standard curve, and the slope and intercept were determined by using computer software (GraphPad Prism 6.0, GraphPad Software - CA, USA). All assays were performed by a single operator.

Linearity

To assess the linearity and sensitivity of the assay, serial dilutions of normal canine plasma pool were used. The pool was diluted 1:25 with PBS/BSA 0.5% washing buffer, and was then serially diluted to achieve the concentrations of 50.0%, 25.0%, 12.5%, 6.25%, 3.12% and 0% of vWF:Ag. The results were plotted and the coefficient of determination (R^2) calculated.

Precision

For intra-assay test, 3 patient samples (dog 1 – hemophilia A; dog 2 – type 1 von Willebrand disease; dog 3 – normal dog) were obtained. Cryoprecipitate (CRYO) and cryosupernatant were also used. vWF:Ag concentrations were repeated 8 times on the same 96-well plate, and coefficients of variation (CVs) were calculated using the OD results. The inter-assay precision was evaluated using the normal CPP, CRYO and cryosupernatant. The vWF:Ag of the samples were determined 8 to 31 times on 96-well plates, and the CVs were calculated. The values for inter-assay precision were represented as percentage of a normal CPP.

vWF:Ag reference range

To determine a reference interval, blood from 220 healthy dogs, without history of hemorrhagic diathesis or thrombocytopenia were obtained. All dogs were submitted to clinical evaluation as described for the CPP dogs. This

population included 118 females and 102 males, from 0.5 to 16 years and several breeds (Supplementary Table 1). Samples were collected, processed and stored as previously described for standard protocols. All samples were submitted to the vWF:Ag assay. The reference limit was determined according to American Society for Veterinary Clinical Pathology (ASVCP) guidelines (FRIEDRICHS et al., 2012).

Coagulation tests

Samples were also submitted to coagulation tests. Activated Partial Thromboplastine Time (aPTT) was determined with commercial kit (PTT automate 5, Diagnostica Stago - NJ, USA). FVIII was quantified by the ability of the patient's sample to correct the aPTT from FVIII deficient plasma (FVIII-deficient plasma, Siemens - BE, Germany) and reported as percentage of a normal canine pool plasma (MISCHKE, 2001). All analyses were performed on a semi-automated coagulometer (STart®, Diagnostica Stago - NJ, USA).

Statistical analyses

vWF:Ag, FVIII:C and APPT values were compared using Spearman correlation coefficient. Also, a Spearman correlation for vWF:Ag and age was made. WF:Ag and FVIII:C were submitted to linear regression. Comparison of vWF:Ag and FVIII:C among breeds was performed including only breeds represented with more than five dogs. One-way ANOVA was followed by Kruskal-Wallis and Dunn's multiple comparison tests for vWF:Ag, and for FVIII:C, one-way ANOVA was followed by Tukey's test. To verify differences of vWF:Ag and FVIII:C between sexes, Mann-Whitney test was performed. The patients were then categorized in three groups (age classes) as follows: young dogs, middle aged dogs, and senior dogs. Since there are differences in longevity among breeds, dogs were considered senior when on the 25% final of the life expectancy of the breed (SALVIN et al., 2012). The young dogs category included dogs with no more than one year old. Dogs between 1 year old and the final 25% of the life expectancy were categorized as middle aged dogs. On this analysis one-way ANOVA followed by Kruskal-Wallis and Dunn's multiple

comparison tests was used. Comparison of FVIII:C between dogs with <50% and >50% vWF:Ag was also made, using Mann-Whitney test. All analyses were executed with commercial software (GraphPad Prism 6.0, GraphPad Software - CA, USA). Significance level was $P = 0.05$.

Results

An ELISA was performed to detect the best combination of antibodies to the assay. The combination which achieved the best signal to noise ratio was the rabbit IgG on the coating step and the mAb vW18 as the secondary antibody. All combinations demonstrated reactivity to human plasma, on different levels. Table 1 shows different reagent combinations and the OD result for CPP samples.

Table 1 – OD results for reagent combination to detect vWF on CPP.

	Capture antibody		
Detection antiboy	Rabbit IgG α-vWF	mAb vW18	mAb vW21
Rabbit IgG α-vWF	0.001	0.003	0.101
mAb vW18	0.361	0.112	0.039
mAb vW21	0.005	-0.001	0.002

Results are expressed as optical density difference between CPP and blank (PBS/BSA 0.5%).

The check board titrations results are shown on tables 2 and 3. The first step was to verify which dilution of the coating reagent and samples would generate the best signals. Increasing concentrations of rabbit IgG α-vWF at coating step did not increase OD. Also, increasing the sample dilution produced little differences on OD. Then, an ELISA to detect suitable dilution for the peroxidase-conjugated mAb vW18 was done, and the results demonstrate a good performance for this antibody at increased concentrations.

Table 2 – Checkerboard titration for serum α-vWF and canine samples.

CAD (µg/mL)	**Sample**									Blank
	CPP			CRYO			Cryossupernatant			
	1:10	1:20	1:40	1:10	1:20	1:40	1:10	1:20	1:40	
0.5	0.384	0.354	0.350	0.458	0.458	0.453	0.177	0.167	0.156	0.099
1.0	0.463	0.406	0.350	0.487	0.506	0.495	0.210	0.200	0.185	0.102
2.0	0.492	0.463	0.444	0.509	0.513	0.537	0.242	0.223	0.210	0.112
4.0	0.552	0.521	0.505	0.595	0.554	0.528	0.300	0.253	0.243	0.120
NC	0.098	0.095	0.095	0.103	0.103	0.104	0.101	0.098	0.095	0.104

Results are expressed as optical density. Peroxidase-conjugated mAb vW18 at 1:2000; NC = negative control (coating buffer); CAD = capture antibody dilution. The shaded area indicates the dilution of capture antibody with enhanced performance, which was then chosen for the further standardization.

Table 3 – Checkerboard titration for mAb vW18.

	mAb vW18				NC
Samples	1:100	1:500	1:1000	1:2000	
CPP	1.055	0.824	0.516	0.345	0.055
CRYO	1.200	0.956	0.629	0.395	0.055
Cryossupernatant	0.325	0.289	0.212	0.143	0.056
Blank	0.173	0.109	0.091	0.076	0.054

Results are expressed as optical density. The micro plate was coated with 1µg/mL of rabbit IgG α-vWF; B = Blank (PBS/BSA 0.5%); NC = negative control (PBS/BSA 0.5%).

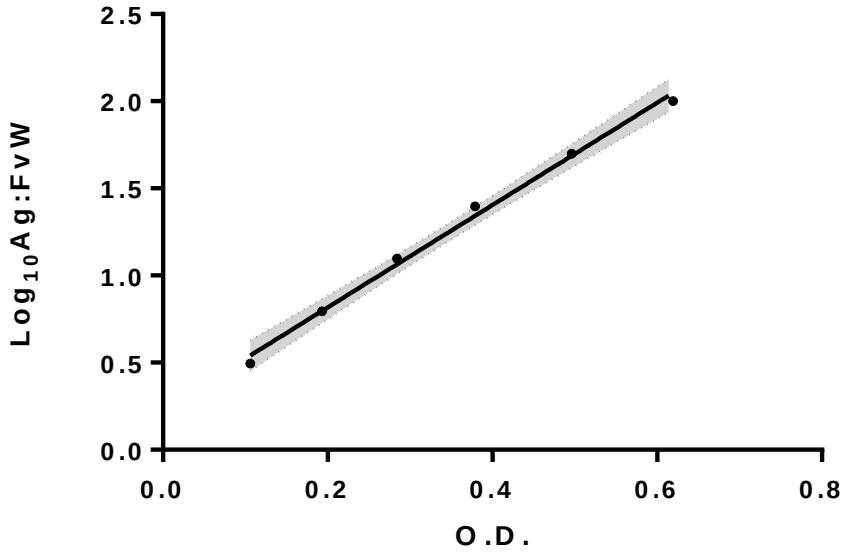

Figure 1 - Linear regression scatter plot after semi-log transformation of vWF:Ag. The canine plasma pool (control) was diluted at 1:25, 1:50, 1:100, 1:200, 1:400 and 1:800 to make the standard curve of vWF:Ag; $R^2 = 0.9946$; $y = 2.963x + 0.2298$.

The R^2 for the linearity from 9 different plates ranged from 0.9442 to 0.9946 (mean 0.9810), with a CV of 1.64%. On figure 1 are reported graphically the linearity results for vWF:Ag. Intra-assay CVs for dogs 01, 02, 03, CRYO, cryosupernatant and normal CPP were 3.64%, 3.17%, 1.83%, 4.54%, 2.34% and

3.24%, respectively (Table 4). The inter-assay CVs for normal CPP, CRYO and cryosupernatant samples were 12.98%, 17.75% and 9.02%, respectively (Table 1). Since the vWF:Ag results were plotted on logarithmic values to achieve a normal distribution, no outliers were detected.

No outliers were identified, and the reference range for the assay was between 24.87 – 224.5%. A total of 47 (21.36%) dogs had vWF:Ag values below 50% (table 6), and 5 dogs (2.27%) had values under the inferior reference range (table 8).

The correlation coefficients (*r*) found between vWF:Ag and FVIII:C, vWF:Ag and APTT and FVIII:C and APTT were 0.418, -0.204 and -0.254, respectively (Table 5). The linear regression coefficient (R^2) of vWF:Ag and FVIII:C was 0.1646. No statistical differences for vWF:Ag or FVIII:C were found among breeds or between sexes. Again, no differences for vWF:Ag among age classes were found, and the *r* for vWF:Ag and age was 0.117. When FVIII:C was compared between dog with vWF:Ag < 50% and > 50%, statistical difference were found ($P = 0.007$).

Table 4 - Intra-assay and inter-assay precision for the vWF:Ag assay.

	Intra-Assay		Inter-Assay[#]	
Sample	Mean ± SD (OD)	CV (%)	Mean ± SD (vWF:Ag%)	CV (%)
Dog 01 (HA)	1.09 ± 0.039[a]	3.64		
Dog 02 (vWD1)	0.58 ± 0.018[a]	3.17		
Dog 03	0.97 ± 0.017[a]	1.83		
CRYO	1.06 ± 0.048[a]	4.54	500.7 ± 88.91[b]	17.75
Cryosupernatant	0.62 ± 0.014[a]	2.34	3.716 ± 0.3354[c]	9.02
Normal CPP	1.00 ± 0.032[a]	3.34	102.5 ± 13.30[d]	12.98

SD: standard deviation; OD: optical density; CRYO: cryoprecipitate; CPP: canine plasma pool; HA: hemophilia A; vWD1: von Willebrand Disease type 1; [a] 8 determinations; [b] samples repeated in 8 assays; [c] samples repeated on 12 assays; [d] samples repeated on 31 assays; [#] samples repeated on different days.

Table 5 - Spearman correlation coefficients for vWF:Ag, FVIII:C and APTT.

	vWF:Ag	FVIII:C	APTT
vWF:Ag	1.000	-	-
FVIII:C	0.418	1.000	-
APTT	-0.204	-0.254	1.000

Table 6 - Dogs with vWF:Ag below 50%.

Breed	N	vWF:Ag* (%)
Basset Hound	1	28.44
Beagle	5	35.98 (27.55 – 45.48)
Belgian Shepherd Dog	3	42.58 (42.50 – 42.69)
Boxer	1	25.20
English Cocker Spaniel	1	33.80
German Shepherd	3	34.25 (21.69 – 41.79)
Giant Schnauzer	3	38.85 (34.22 – 46.83)
Golden Retriever	10	29.57 (20.64 – 41.84)
Great Dane	4	44.66 (40.93 – 47.48)
Labrador Retriever	2	46.78 (45.01 – 48.55)
Lhasa Apso	2	26.50 (25.42 – 27.58)
Miniature Dachshund	2	20.03 (12.43 – 27.63)
Miniature Poodle	5	36.45 (22.34 – 42.51)
Miniature Schnauzer	1	28.71
Saint Bernard	1	46.88
Shih Tzu	1	42.65
Yorkshire Terrier	2	39.25 (32.34 – 46.16)
Total	47	35.28 (12.43 – 48.55)

*Values are represented as means and amplitude for breeds with more than one individual.

Table 7 - vWF:Ag and standard deviation (SD) of breeds with more than five dogs.

Breed	n	vWF:Ag (mean)	SD
Beagle	11	68.41	43.79
Belgian Shepheard	7	60.47	21.08
Boxer	8	107.88	60.97
German Shepheard	11	66.09	23.99
Giant Schnauzer	9	57.38	19.90
Golden Retriever	22	60.36	44.02
Great Dane	18	91.72	39.29
Labrador Retriever	8	79.86	39.84
Lhasa Apso	7	78.79	45.86
Miniature Dachshund	13	98.72	55.49
Miniature Poodle	19	80.54	48.58
Mongrel	17	103.19	51.87
Pinscher	6	123.53	62.13
Shih-Tzu	10	119.00	68.42
Yorkshire Terrier	10	101.21	57.69

Table 8 – Dogs with vWF:Ag below 24.87%.

Breed	vWF:Ag	Gender
Miniature Dachshund	12.43	Female
Golden Retriever	20.64	Female
German Shepheard	21.69	Male
Miniature Poodle	22.34	Male
Golden Retriever	24.46	Male

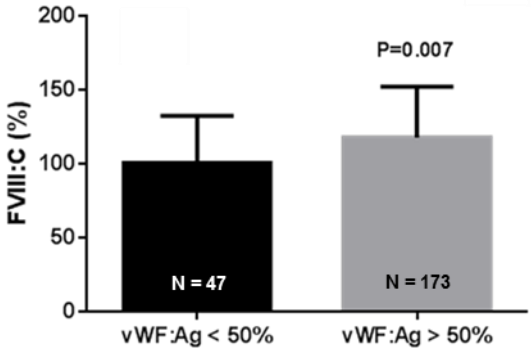

Figure 2. Clotting activity of FVIII of dogs with vWF:Ag above and below 50%.

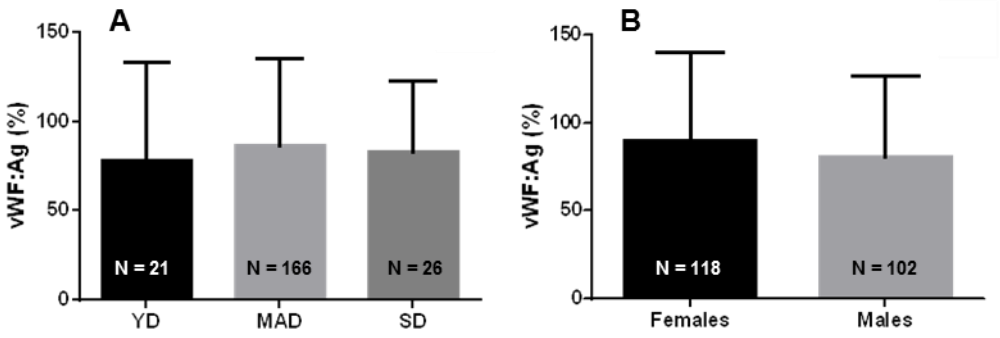

Figure 3. A and B: vWF:Ag among young dogs (YD), middle-aged dogs (MAD) and senior dogs (SD), and between males and females.

Discussion

Since the 90s, the electroimmunodiffusion for canine vWD diagnosis has been replaced by ELISA, which now is the standard assay for vWF quantitation (SLAPPENDEL et al., 1992; BROOKS; CATALFAMO, 2013). However, the lack of availability and high cost, are the main problems for the disease diagnosis at some countries, especially those in underdeveloped or developing continents. Local production of materials and standard protocols are important tools to minimize costs and accelerate the canine vWD diagnosis at these places.

The cross-reactivity property of antibodies allows that, after proper evaluation, antibodies to human proteins may be suitable to detect homologous

protein of animals (DAVIS et al., 1987; TRAHAIR et al., 1995; MIURA et al., 2013). Moreover, ELISA techniques with anti-human vWF has been widely adopted to determine the vWF:Ag determination on canine samples, and several studies have demonstrated that this antibodies may have good performance (BENSON et al., 1991; JOHNSTONE; CRANE, 1991; STOKOL et al., 1997; SATO; PARRY, 1998; JOHNSTONE, 1999). Although human and canine vWF are 86.2% identical at amino acid sequence, residual difference between the proteins can be the cause of the undetectable reaction of vW21 mAb (NICHOLS et al., 2010).

The vWF is a multimeric protein, with repeated domains, which may allow to use the same mAb for vWF:Ag ELISA (DE WIT; VAN MOURIK, 2001; SADLER, 2005b). However, when only vW18 mAb was used both as capture and detection in vWF:Ag assay, a weak reaction was observed, which could be due to epitope competition of the capture and detection antibodies. Since rabbit IgG are a set of distinct immunoglobulins capable of recognizing different epitopes from vWF, a stronger reactivity was expected with these antibodies (FISCHER et al., 1996). However, we cannot generate signal using rabbit IgG anti-vWF as capturing and detecting reagents, probably because the antibodies blocked all epitopes at the capture step (ABBAS, 2015).

Despite the vWF conformation permits mAb-binding vWF:Ag ELISA development, most of the canine vWF:Ag ELISA assays adopt serum α-vWF at least at the capturing step (BENSON et al., 1991; JOHNSTONE; CRANE, 1991; SLAPPENDEL et al., 1992; BENSON et al., 1992; FISCHER et al., 1998; STOKOL; PARRY, 1998; BURGESS; WOOD, 2008). Based on table 1, the combination of antibodies chosen for the vWF:Ag ELISA were rabbit IgG anti-vWF as the capturing antibody and peroxidase-conjugated vW18 mAb as the detecting antibody.

All antibodies were produced using vWF:Ag purified from lyophilized commercial VIII/vWF concentrate, which may elicit a production of antibodies for FVIII. To identify if the chosen reagents (vW18 mAb and rabbit IgG α-vWF) for validation would recognize canine FVIII, we performed the precisions assays with canine hemophilia A sample (0.8% FVIII:C activity). Since we were able to identify a good signal (O.D = 1.09) on hemophilia A sample, this feasibility was

eliminated. In addition, the test shown capability to distinguish samples with low and normal vWF:Ag, as we can see on the CPP and cryosupernatant results.

The linearity between vWF concentration and absorbance in ELISA was obtained by semi-log transformation of vWF:Ag, with R^2 ranging from 0.9442 to 0.9946 (mean 0.9810). This coefficient of determination is adequate to permit a clinical interpretation from ELISA results and is similar to previous reports (SLAPPENDEL et al., 1992; BURGESS; WOOD, 2008).

Precision evaluation consists of a two-prong assessment: intra-assay precision and inter-assay or day to-day variation. A good intra-assay CV should be <10%, but for vWF assays the imprecision in terms of CV may be between 10-20% (MARLAR, 2009). The intra-assay CVs results were acceptable, as previously reports for canine vWF:Ag determination using anti-human vWF antibodies (JOHNSTONE; CRANE, 1991) or anti-canine vWF:Ag (SLAPPENDEL et al., 1992; BURGESS; WOOD, 2008). Inter-assay precision is evaluated by repeating the same specimens on the same instrument. In general, the imprecision for inter-assay studies are greater than observed for intra-assay studies. For some of the more complex assays, the imprecision can increase to a significant 30–40% (MARLAR, 2009). As the assay was standardized to identify canine vWF:Ag deficiencies, the inter-assay results were best for samples with low vWF concentrations, as we can see on table 1. Since there is no international "gold" standard sample for canine vWF:Ag, accuracy studies were not performed (STOKOL, 2012).

To help diagnose vWD with our test we made a reference interval with 220 clinically healthy dogs. The inferior limit of normality found for the test was 24.87%, and only 5 dogs (2.27%) had vWF:Ag results below this limit. In type 1 vWD, critical limits of vWF:Ag concentration can be used as approximate rough estimate of hemorrhagic risk or response to transfusion therapy; however, results do not always correlate with clinical signs (BURGESS et al., 2009). The critical threshold of vWF:Ag (the concentration below which a dog is considered "at risk" of hemorrhage) varies from 25-40% in dog with type 1 vWD, although not every dog with concentrations below this threshold will bleed excessively. So, clinical evidence of hemorrhage due primary hemostasis defect (without thrombocytopenia) is paramount for vWD diagnostic on a patient with

questionable vWF:Ag values (STOKOL, 2012). Despite no clinical signals were found on these five patients, the low values of vWF:Ag supports a vWD diagnose, because clinical expression of vWD is variable, with many affected dogs not presenting any evidence of excessive bleeding. Likewise, heterozygous carrier may have low vWF:Ag values and show no signs of vWD (STOKOL et al., 1995c).

When we use the classical vWF:Ag reference limits, (i.e., 70-180% = normal range for vWF:Ag; 50-69% = borderline normal or indeterminate range for vWF:Ag; <50% = abnormal or carrier range for vWF:Ag), we obtained 21.36% (n=47) dog with values above 50%, represented only by pure breed individuals (Table 6). A previous study performed on specific breeds using the classical classification found a prevalence of 23% (Scottish Terrier), 73% (Doberman Pinscher) and 30% (Shetland Sheepdog) dog with vWF:Ag above 50% (BROOKS et al., 1992). Another studies found, 13% (German Wirehaired Pointers), 23% (Scottish Terriers) and 61% (Doberman Pinscher) dog with the deficiency (STOKOL et al., 1995b; STOKOL et al., 1995c; BROOKS et al., 1996). On the other hand, other researchers found low prevalence of the deficiency on Bernese Mountain dogs (0.62%) (ARNOLD et al., 1997). On one study, a prevalence of 1.43% vWD among a dog population without clinical evidence of hemorrhage was found, which is similar to the human prevalence (RODEGHIERO et al., 1987; WERNER et al., 1993; MATTOSO et al., 2010). Since general recommendations for validation of a test include determination of reference interval, we adopt our limits (24.87 – 224.5%) for vWF:Ag deficiency classification. Still, it's important to keep on mind that vWD diagnostic shouldn't be based on one sample results, and every dog with low values should be recollected to confirm the result (ARNOLD et al., 1997).

Plasma vWF circulates as a complex with factor VIII. In humans, marked secondary decreases in factor VIII coagulant activity may occur with vWD (FAVALORO, 2006). Factor VIII levels in vWD dogs seldom drop more than 20%, and as a result, APTT times in affected dogs are usually normal (JOHNSON et al., 1988; PATHAK, 2004). Since there is a biological relationship between vWF and FVIII, a weak correlation (0.418) was obtained between these factor,

and we were able to detect lower levels of FVIII:C for dog with vWF:Ag below 50% (Fig. 2).

Studies with human vWD demonstrated that age influences on vWF:Ag levels, with higher amounts of the factor in elderly people (GILL et al., 1987). Our study fail to demonstrate this association, with low correlation between vWF:Ag and age, and no age–related differences (Fig. 3A). In addition, no differences between gender were present (Fig. 3B). As only was demonstrated differences on human vWF:Ag when larger populations (>5000 samples) were analyzed, we speculated if an study including a greater number of patient would be able to identify the same vWF behavior on dogs (DAVIES et al., 2012).

No differences were found for vWF:Ag among breeds (Table 7). However, this analysis was made only on 14 pure breeds and mongrel dogs, and the groups weren't standardized (n ranges from 6 to 22 dogs). In addition, it is well documented the influence of ABO group and ethnics on vWF:Ag levels in humans (GILL et al., 1987; SUKHU et al., 2003; DAVIES et al., 2012). Since there may have different frequencies of dog erythrocyte antigen (DEA) among purebreds, an experimental design including vWF:Ag and blood typing on larger dog breeds groups would be capable to elucidate this hypothesis (ARIKAN et al., 2009; IAZBIK et al., 2010; ESTEVES et al., 2011; EKIZ et al., 2011; FERREIRA et al., 2011).

Conclusions

The assay is suitable to detect vWF deficiencies on canine samples, and also can support on type 1 and type 3 classification. However, this assay should not be employed on dogs without clinical evidence of a primary hemostatic disturbance, since healthy dogs may have low vWF:Ag. In addition, aPTT and FVIII:C are not good parameters to help canine vWD diagnosis. This work developed a tool for canine vWD diagnosis and researches and this incorporate mAb technology for vWF:Ag assay. Finally, it is necessary to increase the number of dogs and perform the DEA typing to elucidate the hypothesis of blood type and ethnics influence on the vWF, as we can see in human beings

Conflict of interest statement

None of the authors of this paper has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

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Supplementary Table 1 - Breeds included on reference range for vWF:Ag.

Breed	N	Breed	n
Beagle	11	Great Dane	18
Australian Cattle Dog	1	Irish Setter	1
Basset Hound	2	Labrador Retriever	8
Beagle	11	Lhasa Apso	7
Belgian Shepheard	7	Maltese	2
Bernese Mountain Dog	3	Miniature Dachshund	13
Bichon Frise	2	Miniature Poodle	19
Border Collie	1	Miniature Schnauzer	4
BostonTerrier	1	Mongrel	17
Boxer	8	Pequinese	1
Brazilian Mastiff	1	Pinscher	6
Brazilian Terrier	1	Pit Bull	1
Bulldog	1	Pug	3
Chow Chow	1	Rottweiler	3
Dalmatian	3	Saint Bernard	3
Doberman Pinscher	1	Shih-Tzu	10
Dogue de Bordeaux	1	Weimaraner	2
English Cocker Spaniel	3	West White Highland Terrier	1
German Shepheard	11	White Shepherd	1
Giant Schnauzer	9	Yorkshire Terrier	10
Golden Retriever	22	Total	220

3.3 Artigo Científico 2

VALORES DE REFERÊNCIA DE TEMPOS DE COAGULAÇÃO PARA UMA POPULAÇÃO DE CÃES EM PORTO ALEGRE/RS

Magnus Larruscaim Dalmolin, Camila Serina Lasta, Luciana de Almeida Lacerda, Elisa Rocha de Araújo, Mariana Loner Coutinho, Itabajara da Silva Vaz Junior

Resumo

O tempo de protrombina (TP) e o tempo de tromboplastina parcial ativada (TTPA) são exames rotineiros frente a suspeita de coagulopatias. Estes parâmetros apresentam grandes variações, devido principalmente à diversidade de reagentes e analisadores disponíveis no mercado, além de variações inerentes às diferentes populações estudadas. Existem poucos estudos na literatura veterinária sobre intervalos de referência para testes de coagulação em cães; e para melhor interpretá-los é necessário obter um intervalo de referência da população local, levando em consideração a metodologia a ser utilizada no laboratório. As mensurações do TP (n = 71) e TTPA (n = 258) foram realizadas em uma população local de cães saudáveis, através de coagulômetro semiautomático. O intervalo de referência para TP foi de 6,06 a 9,32 segundos, e o intervalo para o TTPA foi de 15,25 a 24,57 segundos. Os resultados de TP obtidos foram semelhantes a estudos anteriores, já os resultados de TTPA foram diferentes, provavelmente devido a especificidade dos reagentes usados, ou a uma característica da população estudada.

Palavras-chave: hemostasia, tempo de protrombina, tempo de tromboplastina parcial ativada, valores de referência, cães.

Introdução

A hemostasia é um processo fisiológico que tem como objetivos a manutenção da integridade e do fluxo vascular e o controle da hemorragia e da trombose. Os distúrbios da hemostasia são muito comuns em pequenos animais, e consistem em uma série de alterações patológicas que levam o indivíduo a apresentar hemorragia ou trombose. Estas alterações podem ser congênitas ou adquiridas, e os sinais clínicos apresentados podem indicar se é uma deficiência na hemostasia primária, secundária ou ambas (BAKER, 2007; SMITH, 2010).

Em casos de coagulopatias, os pacientes apresentam sinais hemorrágicos como hematomas e hemorragias profundas. Além disso, distúrbios da hemostasia podem ser causados também por deficiências vasculares, hepáticas e renais. A trombose e o tromboembolismo são decorrentes de falhas na inibição da coagulação e no processo final da hemostasia, a fibrinólise; enquanto a coagulação intravascular disseminada (CID) é uma síndrome complexa que inclui alterações em todas as etapas da coagulação (JOHNSTONE, 2002a; JOHNSTONE, 2002b; BAKER, 2007).

Apesar da importância de um histórico detalhado e de um exame clínico acurado, testes laboratoriais são necessários para estabelecer a presença da maioria dos transtornos da hemostasia. A avaliação laboratorial é indispensável na investigação de desordens hemostáticas, mas suas limitações devem ser conhecidas. Os testes TP e TTPA são rotineiramente utilizados para avaliar a atividade geral do mecanismo da hemostasia; entretanto, a identificação de problemas causados pela falta de fatores de coagulação como a deficiência do fator VIII (hemofilia A) passa por exames laboratoriais ainda mais específicos (SMITH et al., 2005; GEFFRE et al., 2010; BARR; MCMICHAEL, 2012; BROOKS; CATALFAMO, 2013).

Geralmente, os testes que avaliam a coagulação baseiam-se na mensuração do tempo decorrido entre ativação de uma ou mais partes da cascata de coagulação e a formação do coágulo, *in vitro*. O TP é um teste sensível para detectar defeitos na via extrínseca (fator VII) e/ou defeitos na via comum (fibrinogênio, fatores II, V e X), apresentando valores prolongados em casos de intoxicação por dicumarínicos, doença hepática, deficiências específicas de fator e CID, enquanto o TTPA identifica anormalidades de coagulação das vias intrínseca - antes da etapa de conversão da protrombina em trombina - e via comum da

coagulação, sendo utilizado como triagem no diagnóstico de hemofilias e doença de von Willebrand, por exemplo (SILVA; HASHIMOTO, 2006; BAKER, 2007; BAUER et al., 2009). A disponibilidade de avaliação laboratorial da hemostasia é muito importante para o diagnóstico e tratamento destas doenças, entretanto, reagentes de uso veterinário não estão disponíveis comercialmente e se faz necessária adaptação de reagentes de uso humano, sendo importante a obtenção de valores de referência de acordo com cada metodologia e população analisada (LOPES et al., 2005; MEDEIROS; BLANCO, 2009). Ainda, em medicina humana são utilizadas metodologias semiautomáticas e automáticas para a determinação dos tempos de coagulação, o que também começa a ocorrer na medicina veterinária (GEFFRE et al., 2010).

O TP foi introduzido como um teste para a protrombina plasmática antes de que muitos fatores de coagulação que participam da reação fossem descobertos. O TP é realizado pela adição de tromboplastina tecidual e Ca^{2+} ao plasma com citrato e então é avaliado o tempo decorrente até a formação do coágulo. O TTPA é realizado pela incubação do plasma com tromboplastina parcial e um ativador de fator XII e subsequente adição de cloreto de cálcio, seguido da cronometragem do tempo para formação do coágulo (LUBAS et al., 2010).

A interpretação do resultado dos testes de coagulação é um processo de análise comparativa, ou seja, os resultados do paciente são comparados com um "intervalo de referência" para a tomada de decisões, tanto no âmbito do diagnóstico quanto do prognóstico e do tratamento (MARLAR, 2009). Assim, a determinação de intervalos de referência é de fundamental para a prática clínica da medicina veterinária e o presente trabalho tem como objetivo determinar valores de referência para TP e TTPA canino, com o uso de reagentes para testes de coagulação em plasma humano, através de método semiautomático, por sistema de detecção de viscosidade.

Materiais e métodos

Foram selecionados 268 cães, machos e fêmeas, sem padrões raciais definidos, sem histórico de hemorragia e/ou trombocitopenia. Além disso, todos os cães foram submetidos a avaliação clínica (ausculta cardiopulmonar, palpação

abdominal, avaliação de mucosas, hidratação corporal e temperatura retal). Após serem considerados clinicamente saudáveis, foram colhidos por sistema a vácuo 2,7 mL de sangue em citrato de sódio a 3,2% (proporção 9:1) para obtenção de plasma. As amostras foram colhidas através de punção da veia jugular externa, cefálica ou safena lateral, após tricotomia e antissepsia local, e centrifugadas a 1500 g por 15 minutos. O plasma separado em alíquotas (3 a 5), que foram congeladas e armazenadas (-30°C) até o momento da análise. Todas as amostras foram obtidas após consentimento dos tutores, e os procedimentos foram aprovados pela Comissão de Ética no Uso de Animais da Universidade Federal do Rio Grande do Sul (CEUA-UFRGS) – sob o número de registro 24925.

Para as determinações do TP foram utilizadas amostras de 71 cães. Estas foram descongeladas em banho a 37°C por um a três minutos, imediatamente antes de serem analisadas. As amostras de plasma foram incubadas a 37°C por um minuto, e então a coagulação iniciada pela adição de cálcio, tromboplastina tecidual e um inibidor de heparina (Neoplastine® CI Plus – Diagnóstica Stago). O tempo foi cronometrado automaticamente até a coagulação da amostra.

Para que a distribuição dos resultados do TTPA atingisse a normalidade, foram utilizadas amostras de 258 cães, descongeladas como previamente descrito. O plasma foi incubado a 37°C por três minutos com cefalina e sílica em reagente tamponado (PTT Automate 5 – Diagnóstica Stago), e a partir da adição de $CaCl_2$ 25 mM, o tempo até a coagulação da amostra foi cronometrado. Todas as análises de coagulação foram realizadas conforme instruções do fabricante, em coagulômetro semiautomático STart 4 Hemostasis Analyzer (STart® - Diagnostica Stago) pelo método de coagulação - sistema de detecção de viscosidade.

Os intervalos de referência foram determinados conforme ASVCP (*American Society for Veterinary Clinical Pathology*), após a identificação de *outliers* (FRIEDRICHS et al., 2012). A distribuição dos dados foi avaliada pelo teste de normalidade de D'Agostino & Pearson. Os limites de referência para TP foram determinados a partir dos valores médios da população normal ± 2 desvios padrão. Para o TTPA, o intervalo de referência foi construído após classificação dos dados, determinação dos percentis 2,5 e 97,5% e obtenção do intervalo de

confiança de 95%. As análises foram executadas em software comercial (GraphPad Prism 6.0, GraphPad Software, CA, EUA).

Resultados

Segundo ASVCP (FRIEDRICHS et al., 2012), quando a amostragem é superior a 120 indivíduos, recomenda-se que o intervalo de referência seja determinado através de métodos não paramétricos, com 95% de confiança. Assim, foi obtido um intervalo de referência do TTPA 15,25 a 24,57 segundos. Para a determinação do intervalo do TP em um grupo de 71 indivíduos foi seguida a recomendação de se utilizar a média ± dois desvios padrão, e o resultado obtido foi de 6,06 a 9,32 segundos. Ambas as distribuições passaram no teste de normalidade e não foram identificados valores atípicos na população estudada. Os intervalos de referência para TP e TTPA estão apresentados na tabela 1. As distribuições dos tempos de coagulação da amostra populacional de referência estão apresentadas na figura 1.

Tabela 1 – Estatística descritiva e limites de referência para TP e TTPA para a população de cães avaliada, com intervalo de confiança de 95%.

	TP (s)	TTPA (s)
Média (desvio padrão)	7,69 (+/-0,81)	19,44 (+/-2,33)
Limite inferior de referência	6,06	15,25
Limite superior de referência	9,32	24,57
Mínimo - máximo	5,30 – 10,00	13,00 – 26,60
N	71	258

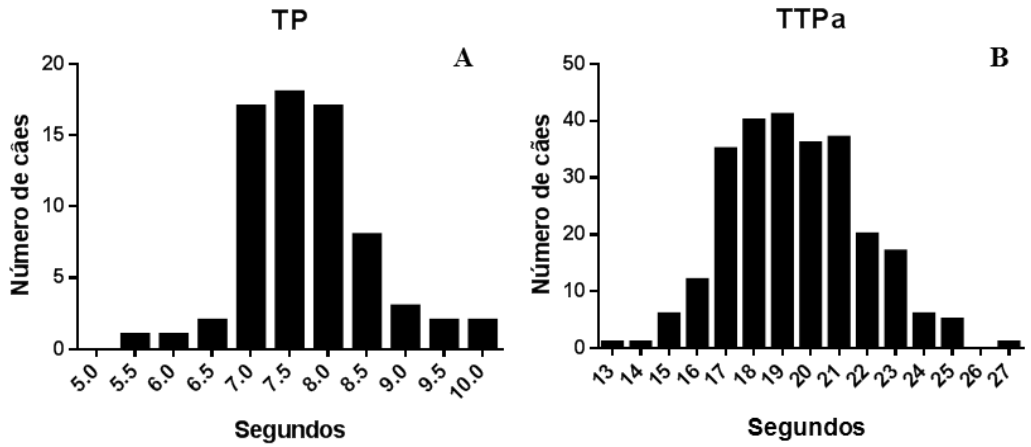

Figura 1. Histograma de distribuição dos valores de TP (A) e TTPa (B) da população de cães.

Discussão

O TP e o TTPA fazem parte dos testes de coagulação de triagem e são referidos como parâmetros básicos na avaliação da hemostasia secundária (BROOKS; CATALFAMO, 2013). Apesar da transferência de intervalos de referência ser amplamente utilizada na patologia clínica (principalmente em parâmetros de bioquímica clínica), a determinação de intervalos próprios em cada laboratório é a forma mais indicada para se interpretar resultados de exames laboratoriais (MINEO; GARABED, 2007; MARLAR, 2009; GEFFRE et al., 2011). Além disso, diferentes resultados para parâmetros de coagulação são descritos quando se avaliam diferentes grupos de cães e quando se utilizam diferentes reagentes e métodos – manuais, semiautomáticos, automáticos, ou testes rápidos (EVANS; FLYNN, 1992; TSENG et al., 2001; LOPES et al., 2005; HENNAN et al., 2005; GEFFRE et al., 2010; NIELSEN et al., 2011).

A maior atividade dos fatores de coagulação envolvida nas vias extrínseca e comum faz com que os intervalos de referência descritos para o TP sejam mais baixos e estreitos que os intervalos para TTPA (MISCHKE; NOLTE, 1997; LOPES et al., 2005; RIZZO et al., 2008; GEFFRE et al., 2010; LUBAS et al., 2010). Na tabela 2 pode ser observado que os intervalos de referência para TP canino encontrados neste estudo são semelhantes aos citados na literatura, mesmo quando se variam os instrumentos e reagentes. Isto pode ser atribuído ao fato de que os reagentes de TP variam pouco, pois a tromboplastina tecidual e o Ca^{2+}

continuam sendo os reagentes base para o teste, independentemente do kit ou equipamento. Basicamente, existem três fontes de tromboplastina para os reagentes de TP: fator tecidual recombinante, tromboplastina tecidual (proveniente de cérebro de coelho e placenta humana) e tromboplastina combinada (tromboplastina de cérebro de bovino diluída em fração de fibrinogênio adsorvida a plasma bovino). O Ca^{2+} pode ser adicionado ao reagente durante a manufatura ou imediatamente antes do teste, dependendo do reagente (LUBAS et al., 2010).

Por outro lado, os reagentes de TTPA são mais diversificados e menos padronizados que os reagentes de TP. Os ativadores utilizados mais comumente são o ácido elágico, o caulim, a sílica micronizada e o celite. Todos esses compostos são usados nos diferentes reagentes de TTPA e possuem a mesma função de ativação do mecanismo de coagulação. Os fosfolipídios são substitutos plaquetários e aceleram as reações. As fontes de fosfolipídios são cérebro de coelho, cefalina (cérebro de coelho desidratado), cérebro de bovino e soja. Usualmente são usadas misturas com diferentes tipos de fosfolipídios, incluindo a fosfatidilserina negativamente carregada. É a combinação do ativador de contato, fosfolipídio, molaridade do cloreto de cálcio, força iônica, sistema de tampões e estabilizantes que determina as propriedades do reagente (LUBAS et al., 2010). Na tabela 3 pode ser observada a maior variação nos intervalos de referência descritos para o TTPA de cães, que deve-se principalmente às variações dos ativadores utilizados em cada reagente (caulim e ácido elágico). Pelo conhecimento dos autores, não há intervalo de referência para TTPA em cães com reagentes que adotam a sílica como ativador, e isto pode justificar o maior intervalo para este parâmetro de coagulação observado neste trabalho. Além disso, este maior valor de TTPA pode ser uma característica específica da população de cães avaliada.

Tabela 2 – Intervalos de referência descritos na literatura para TP em cães.

Número de amostras	Equipamento	Reagente	IR (s)
56	STA Compact	STA Neoplastine Plus	5,7 – 8,0[a]
139	STA Satellite	STA Neoplastine CI Plus	6,9 – 8,8[b]
60	STA-R Evolution	STA Neoplastine CI Plus	7,1 – 9,2[b]
50	Schnitger & Gross	Thromborel S	5,7 – 7,1[c]
50	Schnitger & Gross	Neoplastine Plus	6,6 – 8,6 [c]
50	Schnitger & Gross	Thromboplastin-M	6,5 – 8,5 [c]
50	Schnitger & Gross	Thromboplastin-IS	6,3 – 7,3 [c]
50	Schnitger & Gross	Innovin	5,5 – 7,3 [c]
40	Manual	HemoStat Thromboplastin-SI	4,0 – 9,6[d]
71	Start 4	Neoplastine CI Plus	6,0 – 9,3[e]

IR: intervalo de referência. [a] (BAUER et al., 2009); [b] (GEFFRE et al., 2010); [c] (MISCHKE; NOLTE, 1997); [d] (LOPES et al., 2005); [e] (DALMOLIN et al., 2014).

Tabela 3 – Intervalos de referência descritos na literatura para TTPA em cães.

Número de amostras	Equipamento	Reagente	IR (segundos)
56	STA Compact	STA APTT Kaolin	10,0 - 14,3[a]
139	STA Satellite	STA Cephascreen	13,1 – 17,2[b]
60	STA-R Evolution	STA Cephascreen	12,9 – 17,3[b]
40	Manual	HemoStat aPTT-EL	11,9 – 18,3[c]
258	Start 4	PTT Automate 5	15,2 – 24,5[d]

IR: intervalo de referência. [a] (BAUER et al., 2009); [b] (GEFFRE et al., 2010); [c] (LOPES et al., 2005); [d] (DALMOLIN et al., 2014).

Conclusão

Os reagentes de uso humano podem ser utilizados em amostras caninas, desde que padronizados para tal fim e que sejam obtidos e aplicados valores de referência para a espécie. Os valores de TP e TTPA neste estudo podem ser utilizados como valores referência em laboratórios locais que utilizem a mesma metodologia empregada. Os resultados de TTPA foram diferentes daqueles publicados previamente, devido a utilização de outros reagentes, ou a uma característica da população estudada.

Conflito de Interesses

Nenhum dos autores possui relação pessoal ou financeira com outras pessoas ou organizações que poderiam, inapropriadamente, influenciar ou enviesar o conteúdo deste trabalho.

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3.4 Relato de Caso

DOENÇA DE VON WILLEBRAND TIPO 1 GRAVE EM CÃO DA RAÇA SÃO BERNARDO – APRESENTAÇÃO CLÍNICA E PERFIL DE HEMOSTASIA

Magnus Larruscaim Dalmolin, Camila Serina Lasta, Luciana de Almeida Lacerda, Vanessa Camargo, Mariana Loner Coutinho, Itabajara da Silva Vaz Jr

Resumo

A DvW resulta de uma deficiência quantitativa e/ou qualitativa do FvW. É classificada como um defeito extrínseco à plaqueta, em que a falha da resposta hemostática é atribuída à deficiência de um fator plasmático necessário para a função plaquetária normal. Neste trabalho é relatado um caso de doença de von Willebrand canina. Um cão da raça São Bernardo, fêmea, seis meses de idade, foi atendido em hospital veterinário de ensino devido a uma intensa epistaxe bilateral. Ao exame clínico observaram-se mucosas hipocoradas, desidratação de 8%, taquicardia (148 bpm), hipertermia (40,5°C), apatia, dispneia grave, estertores e condição corporal magra. O hemograma identificou anemia macrocítica hipocrômica grave. A avaliação da hemostasia apresentava um painel de coagulação e contagem de plaquetas dentro dos valores de referência. O tempo de sangramento da mucosa bucal apresentava-se prolongado (17 minutos). O paciente recebeu transfusão de concentrado de eritrócitos e plasma fresco congelado, mas veio a óbito 18 horas após a internação. O fator VIII e o fator de von Willebrand apresentavam-se diminuídos (43% e 4%, respectivamente).

Palavras-chave: fator de von Willebrand, hemorragia, transfusão sanguínea, doença congênita, canino.

Introdução

A doença de von Willebrand (DvW) resulta de uma deficiência quantitativa e/ou qualitativa do fator de von Willebrand (FvW). O FvW é uma glicoproteína

plasmática multimérica que possui importantes funções no processo da hemostasia (SADLER, 2005a). Após injúria vascular, o FvW liga-se ao colágeno subendotelial e passa por mudanças estruturais que facilitam a sua interação com a glicoproteína de membrana plaquetária Ib, permitindo a adesão das plaquetas ao local de hemorragia. O FvW também participa da agregação plaquetária através da interação com as proteínas de membrana plaquetária IIb/IIIa. Devido a sua relação com o fator VIII (o FvW interage com o fator VIII, estabilizando o mesmo), em alguns pacientes a DvW está associada a uma deficiência plasmática de fator VIII (STOKOL et al., 1995a; SMITH, 2010).

A DvW é a doença hemorrágica hereditária mais comum em cães, e afeta mais de 50 raças. É particularmente comum nas raças Dobermann, Terrier Escocês e Pastor de Shetland, e é transmitida como uma herança autossômica (JOHNSTONE; CRANE, 1981; RAYMOND et al., 1990; BROOKS et al., 1992; STOKOL et al., 1995b; STOKOL et al., 1995c; RIEHL et al., 2000). É classificada em três tipos, de acordo com sua patofisiologia: DvW tipo 1 (deficiência parcial do FvW), tipo 2 (deficiência qualitativa do FvW) e tipo 3 (deficiência absoluta do FvW). Os três tipos já foram identificados em cães, sendo o tipo 1 de maior ocorrência (STOKOL et al., 1995b; STOKOL et al., 1995c; VAN DONGEN et al., 2001; JOHNSTONE, 2002a; KRAMER et al., 2004; BROOKS; CATALFAMO, 2010; STOKOL, 2012; GAVAZZA et al., 2012).

A DvW se manifesta com sinais clínicos típicos de um transtorno de hemostasia primária, com hemorragias principalmente nas superfícies mucosas – gengivorragia, epistaxe, melena, hematúria e hemorragia uterina. Algumas vezes, hemorragia prolongada após cirurgia ou trauma (incluindo corte de unhas) pode ser o primeiro sinal apresentado (DODDS, 1970; DODDS, 1975; JOHNSTONE; CRANE, 1981; BROOKS et al., 1992; BROOKS et al., 2001). Outras manifestações clínicas observadas na DvW incluem claudicação, hemorragia intracraniana e cicatrização deficiente (STOKOL, 2012). O presente trabalho visa descrever a abordagem sistemática para o diagnóstico de um caso de DvW tipo 1.

Descrição do caso

Uma cadela da raça São Bernardo, seis meses de idade e 17 kg foi atendida em Hospital Veterinário com epistaxe bilateral contínua (Figura 1), apatia, hiporrexia e melena. Ao exame clínico observaram-se mucosas hipocoradas, desidratação de 8%, taquicardia (148 bpm), hipertermia (40,5°C), apatia, dispneia grave, estertores e condição corporal magra. Uma amostra de sangue com anticoagulante ácido etilenodiamino tetracético dipotássico (EDTA K2, BD Brasil, São Paulo, Brasil) foi colhida para hemograma e teste de compatibilidade sanguínea e outra em anticoagulante citrato de sódio 3,2% (BD Brasil, São Paulo, Brasil) para testes de coagulação.

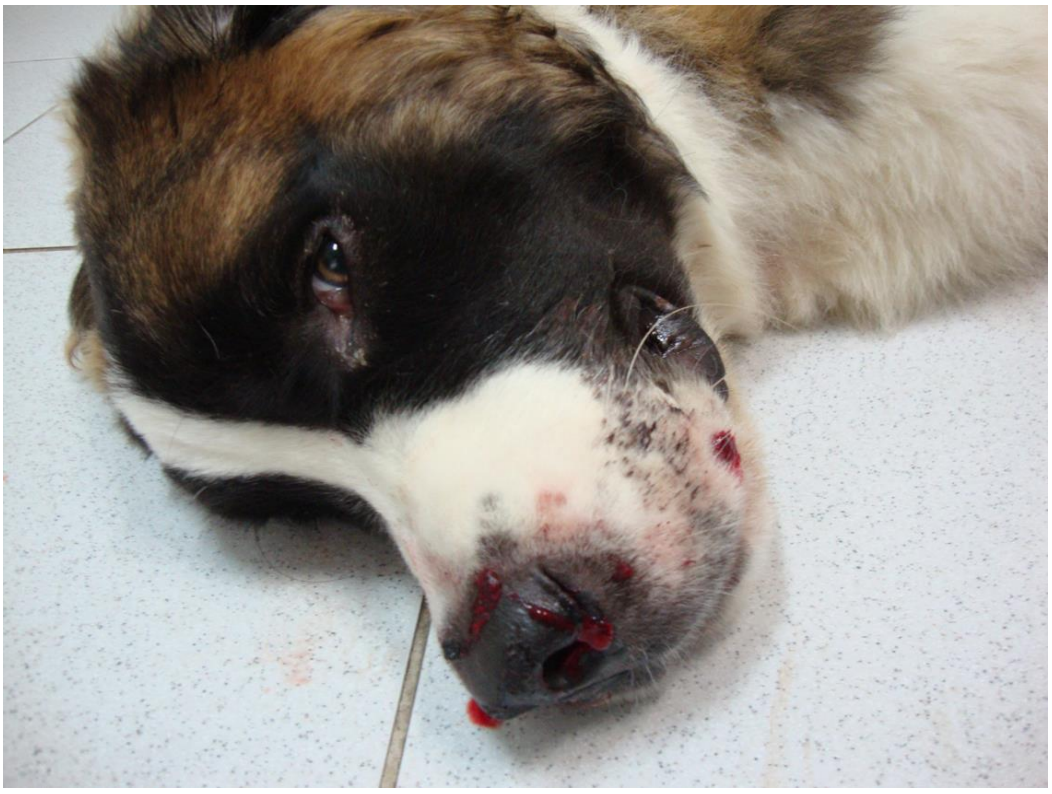

Figura 1: Cão da raça São Bernardo, apresentando epistaxe contínua.

A avaliação laboratorial demonstrou anemia grave (hematócrito de 13% e hemoglobina de 3,7 g/L) macrocítica e hipocrômica, leucocitose neutrofílica e hipoproteinemia; TP, TTPA e contagem de plaquetas dentro dos valores normais para a espécie (Tabela 1). O tempo de sangramento da mucosa bucal (TSMB) foi

determinado conforme Sato e colaboradores (2000), e apresentou-se prolongado (17 minutos). Uma alíquota de plasma em citrato de sódio 3,8% foi armazenada a -70°C para a determinação do antígeno FvW (Ag:FvW) e da atividade coagulante do fator VIII (FVIII:C).

Após prova de compatibilidade através do método da hemaglutinação em tubo de ensaio (WARDROP, 2010), foram administrados 15mL/kg de concentrado de hemácias para correção da anemia. Para promover a hemostasia, plasma fresco congelado foi administrado na dose de 15mL/kg, porém o paciente apresentou piora do quadro respiratório e veio a óbito após 18 horas da internação.

Tabela 1 - Resultados dos exames de hemostasia do paciente

Exame	Resultado	Valores de referência*
TP (s)[#]	7	< 10
TTPA (s)[#]	17	10 – 20
TSMB (s)	1020	128 – 186
Plaquetas (x10³/µL)	318	200 – 500
Concentração do Ag:FvW (%)[§]	4	< 50: deficiente
		50 – 70: suspeito
		> 70: livre
Atividade do FVIII (%)[$]	43	60 – 140

* BROOKS, 2010; BROOKS; CATALFAMO, 2010; LUBAS et al., 2010; RIZZI et al., 2010; [#] Metodologia: coagulação; [§] Metodologia: ELISA (enzyme-linked immunosorbent assay); [$] Metodologia: TTPA modificado com plasma deficiente em FVIII.

O Ag:FvW e FVIII:C do paciente foram determinados em relação a um *pool* de plasma canino normal. Para a confecção do *pool* foram colhidos sangue em anticoagulante citrato de sódio 3,8% de 24 cães clinicamente saudáveis. O *pool* foi confeccionado adicionando-se volumes iguais de cada plasma, separado em alíquotas e armazenado a -70°C até o momento das análises. Esta amostra foi

arbitrariamente considerada com 100% de FvW e de FVIII:C. O Ag:FvW foi determinado por ELISA (DALMOLIN et al., dados não publicados), e a mensuração do FVIII:C foi realizada conforme previamente descrito (BENSON et al., 1991; JOHNSTONE; CRANE, 1991; BENSON et al., 1992; MISCHKE, 2001), em analisador semiautomático STart® (Diagnóstica Stago). O paciente apresentou um valor de 4% de Ag:FvW e 43% de FVIII:C

Discussão

Testes de triagem como contagem de plaquetas, TP e TTPA normais em paciente jovem com histórico de sangramento sugerem distúrbio de função plaquetária (BARR; MCMICHAEL, 2012; BROOKS; CATALFAMO, 2013). Neste caso, a contagem de plaquetas e o perfil de coagulação do paciente apresentavam-se normais, indicando que o distúrbio hemorrágico não estava ligado a coagulopatia ou trombocitopenia. Uma característica importante da DvW é o prolongamento do TSMB. O resultado deste exame demonstrou um valor muito prolongado (17 minutos), mas como este teste pode estar alterado na DvW e em trombocitopatias, exames confirmatórios são necessários para o diagnóstico definitivo. Além disso, deve-se ter cautela ao interpretar o TSMB em pacientes anêmicos, pois a desmarginalização das plaquetas pode induzir um prolongamento do resultado do teste (DEROSSI; GLICK, 1996; SATO et al., 2000; HEDGES et al., 2007; BONEU; FERNANDEZ, 2012).

As principais complicações da DvW são as hemorragias, que dependendo da intensidade e do local podem levar a anemias graves, hipóxia tecidual, hipovolemia, hipoproteinemia ou comprometimento de sistemas como SNC ou urinário, por exemplo (RAYMOND et al., 1990; BROOKS et al., 1992; BROOKS; CATALFAMO, 2010). A severa redução do hematócrito e das proteínas plasmáticas neste caso demonstra a extensão da hemorragia, que quando externa leva a uma perda importante de proteínas e ferro (NAIGAMWALLA et al., 2012)

Anemias hemorrágicas são classicamente regenerativas, e apresentam-se inicialmente macrocíticas e hipocrômicas, pois os depósitos de ferros são geralmente suficientes. Quando a perda sanguínea não é controlada, com o tempo

a anemia pode se apresentar normocítica e normocrômica e mais tarde microcítica e hipocrômica - quando os estoques de ferro são depletados, diminuindo a capacidade de regeneração eritróide, pois para cada mililitro de sangue perdido, perde-se 0,5 mg de ferro (TVEDTEN, 2010; MILLS, 2012). O paciente apresentava epistaxe bilateral contínua, sem traumatismo aparente ao exame clínico. A severidade, o tipo de anemia (macrocítica e hipocrômica) e a hipoproteinemia refletem um processo agudo com duração de mais de 3 dias, o tempo mínimo para se observar uma resposta regenerativa na anemia hemorrágica (MCCOWN; SPECHT, 2011; NAIGAMWALLA et al., 2012). O aspecto das fezes indicava hemorragia gastrointestinal concomitante, o que contribui para a perda externa de ferro e proteínas.

A manifestação clínica da DvW é variável e depende de diversos fatores. A extensão da deficiência e o tipo de DvW são as variáveis mais importantes para determinar a probabilidade e a severidade da hemorragia. Como regra geral, quanto menor a concentração de FvW maior a probabilidade de hemorragia (JOHNSTONE, 2002a; STOKOL, 2012; BOUDREAUX, 2012). No presente caso, a baixa quantidade de FvW (4%) foi determinante para a ocorrência de sangramentos severos. O resultado do Ag:FvW demonstrou que o paciente apresentava uma deficiência do tipo I, a forma mais comum da doença, caracterizada por uma deficiência quantitativa parcial grave na concentração da proteína com funções normais dos multímeros.

O FVIII é uma proteína de coagulação essencial para a fase de propagação da hemostasia (SMITH, 2009). É sintetizado no fígado e no sistema retículo-endotelial e uma parcela é armazenada associada ao fator de von Willebrand nos corpúsculos de Weibel-Palade nas células endoteliais e nos grânulos α plaquetários. A ligação ao FvW aumenta a estabilidade plasmática e intracelular do FVIII, o que faz com que em alguns pacientes a deficiência do FvW seja associada a baixos níveis circulantes de FVIII, o que pode resultar em leve prolongamento do TTPA (SMITH, 2010; BARR; MCMICHAEL, 2012). Apesar do resultado de FVIII:C (43%) do paciente apresentar-se abaixo dos valores de referência, não foi observado um prolongamento no TTPA, pois isto só ocorre quando algum fator de coagulação está abaixo de 30% (STOKOL et al., 1995a; STOKOL et al., 1997). Estes resultados demonstram que a redução dos níveis de

FvW afetou a estabilidade do FVIII:C do cão, mas não o suficiente para induzir um prolongamento do TTPA.

De acordo com o quadro clínico respiratório e com os achados hematológicos foi iniciado tratamento para infecção pulmonar concomitante. O quadro respiratório, associado à anemia contribuiu para a gravidade da hipóxia do paciente, e provavelmente este fato levou o mesmo ao óbito. Ainda, aspiração de sangue pode ter contribuído para o colapso respiratório.

O tratamento da DvW é paliativo, e o objetivo central é a prevenção ou o controle da hemorragia. O produto de escolha para prevenir ou cessar a hemorragia é o crioprecipitado, uma forma concentrada de FvW e FVIII. Este hemocomponente fornece grandes quantidades destes fatores em um pequeno volume, e está associado a menores efeitos colaterais. Além disso, também é mais efetivo para aumentar a concentração do Ag:FvW e encurtar o TSMB (CHING et al., 1994; STOKOL et al., 1997; STOKOL; PARRY, 1998; STOKOL, 2012). Plasma fresco e plasma fresco congelado são boas alternativas ao crioprecipitado, mas estão associados a uma maior ocorrência de reações transfusionais não hemolíticas (GIBSON; ABRAMS-OGG, 2012). A desmopressina, um medicamento que induz a liberação do FvW armazenado nas células endoteliais também pode ser utilizada em pacientes com DvW tipo 1. No entanto, os melhores resultados são alcançados com a infusão de produtos plasmáticos que contenham elevadas concentrações de FvW (BROOKS; CATALFAMO, 2010; STOKOL, 2012). No caso deste paciente, optou-se pela administração de plasma fresco congelado devido à indisponibilidade de crioprecipitado no dia da internação.

Conclusões

Doenças hereditárias devem constar no diagnóstico diferencial de pacientes jovens com distúrbios hemostáticos. Testes de triagem como contagem de plaquetas, TP e TTPA normais em paciente jovem com histórico de sangramento sugerem distúrbio de função plaquetária. A determinação do Ag:FvW é um teste essencial para pacientes com histórico de diátese hemorrágica sem coagulopatia e/ou trombocitopenia e pode ser conclusivo para o diagnóstico de DvW.

Diagnóstico precoce, intervenção terapêutica adequada e reposição de FvW são essenciais para o controle da DvW. Com base nos achados clínicos e exames laboratoriais o paciente foi diagnosticado com DvW tipo I.

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Nenhum dos autores possui relação pessoal ou financeira com outras pessoas ou organizações que poderiam, inapropriadamente, influenciar ou enviesar o conteúdo deste trabalho.

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