Development of a collagen-binding activity assay as a screening test for type II von Willebrand disease in dogs

Elizabeth Peet Sabino, BS; Hollis N. Erb, DVM, PhD; James L. Catalfamo, PhD

Objective—To develop an assay to measure canine von Willebrand factor (vWF):collagen-binding activity (CBA) to screen for type 2 von Willebrand disease (vWD) in dogs.

Sample Population—293 plasma samples submitted for analysis of canine vWF antigen (vWF:Ag) and 12 control plasma samples from dogs with inherited type 2 or 3 vWD.

Procedure—Bovine collagens were evaluated for suitability as binding substrate for vWF. Assay sensitivity to depletion, proteolytic degradation, or a genetic deficiency of high–molecular-weight vWF were determined. Amounts of vWF:Ag and vWF:CBA were measured. The ratio of vWF:Ag to vWF:CBA was used to discriminate between type 1 and type 2 vWD.

Results—An assay for canine vWF activity was developed by use of mixed collagen (types I and III). When vWF:Ag was used to subtype vWD, 48% of the dogs were classified as clinically normal, 9% as indeterminate, and 43% as type 1 vWD. Inclusion of vWF activity resulted in reclassification of 5% of those identified as type 1 to type 2 vWD. However, vWF:CBA of the reclassified dogs was not consistently abnormal, a finding compatible with acquired type 2 vWD. Some Doberman Pinschers had lower antigen-to-activity ratios than other breeds with type 1 vWD, suggesting that Doberman Pinschers have more functional circulating vWF.

Conclusions and Clinical Relevance—Analysis of canine vWF activity should be included among the vWF-specific assays used to confirm type 2 vWD. The prevalence of inherited forms of type 2 vWD in screened dogs is lower than acquired forms that can result secondary to underlying disease. (Am J Vet Res 2006;67:242–249)

Von Willebrand factor is essential for platelet adhesion and hemostatic plug formation at sites of vascular injury. It circulates in plasma as a series of identical subunits that are linked through disulfide bonds to form multimers limited in size by the cleavage (ie, trimming) action of ADAMTS13, the major cleaving protease for vWF. Each vWF subunit contains binding sites for collagen, platelet adhesion receptors, and coagulation factor VIII. Under conditions of high shear, vWF acts as a molecular bridge linking platelet receptors with protein components of the subendothelium (including collagen types 1, III, and VI). The major binding site for collagen resides in the A3 domain of vWF, whereas the A2 domain contains the major platelet-binding site. Both sites are flanked by the ADAMTS13 cleavage site. When bound to its platelet receptor, vWF becomes more susceptible to ADAMTS13 cleavage, which promotes feedback inhibition of platelet adhesion.

The vWF multimers range from 500 to 20,000 kd. Repetition of A3 domains within vWF facilitates high-affinity binding to collagen and promotes the tight anchoring of a platelet plug at an injury site. In addition, vWF functions as a carrier protein for coagulation factor VIII. This protects coagulation factor VIII from proteolytic degradation, prolongs its half-life in circulation, and localizes coagulation factor VIII to sites of vascular trauma, where it acts to accelerate the rate of thrombin generation and clot formation.

Quantitative or functional abnormalities in vWF result in vWD, which is the most common inherited bleeding disorder in humans and dogs. The vWD phenotype in both species is heterogeneous and diagnosed by use of established clinical and laboratory criteria. Currently, vWD is classified into 3 major types on the basis of partial (type 1) or severe (type 3) reduction in the plasma vWF concentration or an abnormality in vWF function (type 2). In dogs, a single type is generally predominant within a breed. In humans, vWD can be further divided into subtypes on the basis of differences in platelet concentration of vWF. In dogs with vWD, assignment of subtypes is precluded by the virtual lack of vWF in platelets.

Approximately 75% to 80% of human patients with hereditary vWD are classified as type 1, 15% to 20% are classified as type 2, and < 0.001% are classified as type III. Relative frequencies have not been established for dogs. However, > 50 breeds have been identified with type 1 vWD, 10 with familial or sporadic type 3 vWD, and 2 with familial type 2 vWD.

Investigators have used canine vWF:Ag ELISAs to detect type 1 or type 3 vWD with high fidelity. However, specific identification of type 2 vWD requires...

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**Table 1**

<table>
<thead>
<tr>
<th>ADAMTS13</th>
<th>Disintegrin and metalloproteinase with a thrombospondin type 1 motif</th>
</tr>
</thead>
<tbody>
<tr>
<td>vWF</td>
<td>von Willebrand factor</td>
</tr>
<tr>
<td>vWD</td>
<td>von Willebrand disease</td>
</tr>
<tr>
<td>vWF:Ag</td>
<td>vWF antigen</td>
</tr>
<tr>
<td>RCoF</td>
<td>Ristocetin cofactor</td>
</tr>
<tr>
<td>vWF:CBA</td>
<td>vWF collagen-binding activity</td>
</tr>
<tr>
<td>CV</td>
<td>Coefficient of variation</td>
</tr>
</tbody>
</table>
inclusion of a functional vWF assay in the testing protocol or analysis of the size distribution of multimers by use of SDS–agarose gel electrophoresis.2 Because of its complexity, the latter generally is used as an adjunct to other functional assays to confirm or define abnormal vWF structure.

The RCoF assay is the method most commonly used to assess the functional activity of human vWF.24 It has limited use in evaluation of vWF activity in dogs because of interfering substances in canine plasma.25,26 In human medicine, the collagen-binding assay has supplanted the RCoF assay as the functional assay of choice.27 The collagen-binding ELISA developed in 1 study28 represents a major advance in measurement of canine vWF activity. However, use of that ELISA as a diagnostic tool is limited by a requirement for extraction of collagen from freshly dissected canine tendons. The purpose of the study reported here was to develop a readily standardized assay to measure canine vWF:CBA and evaluate use of the assay for the diagnosis and classification of vWD in dogs.

## Materials and Methods

### Sample population

Citrated plasma samples (n = 293) obtained from purebred or mixed-breed dogs and submitted to the Comparative Coagulation Center of the Animal Health Diagnostic Center between March 14, 2001, and January 23, 2002, for routine analysis of canine vWF:Ag were analyzed for vWF:CBA. Plasma samples submitted for vWD screening included information on the dog’s age, sex, breed, and clinical history. The sample population also included 12 control plasma samples from dogs (2 German Shorthaired Pointers and 2 German Wirehaired Pointers) with confirmed hereditary type 2 VWD or dogs (3 Shetland Sheepdogs and 5 Scottish Terriers) with type 3 VWD.30 Submitted specimens were excluded from the study when the plasma contained clots, clot fragments, or substantial hemolysis or had a prolonged thrombin clotting time.

### Collagen preparations

Collagens from various sources were evaluated for their ability to bind canine vWF when immobilized to polystyrene microtiter plate. Bovine type I tendon collagen was extracted by use of mildly (0.02M acetic acid) or moderately (0.52M acetic acid) acidic conditions, essentially as reported elsewhere.31 The concentration of collagen obtained in extracts for mildly acidic extraction conditions was 0.16 mg/mL, whereas the concentration was 1.28 mg/mL for moderately acidic extraction conditions. To maximize recovery of acid-soluble collagen, extracts were prepared at 5°C by use of a tissue homogenizer. A 0.3% (wt/vol) aqueous solution of pepsin-solubilized bovine dermal collagen (95% type I collagen and 5% type III collagen) in 0.1% acetic buffer (pH, 2.0) was purchased. Bovine collagen also was purchased as a sterile solution of pepsin-solubilized dermal collagen (mixed types I and III with 95% to 98% type I); this solution had a concentration of 3.1 mg/mL in 0.012N HCl (pH, 2.0).

### ELISA for measurement of vWF

Plasma vWF concentration was measured by use of a double-sandwich antibody ELISA with monoclonal antibodies specific for canine vWF:Ag, as described elsewhere.32 For the collagen-binding assay, microtiter plates were coated with collagen preparations diluted in 0.05M sodium carbonate buffer (pH, 9.6) and incubated at 18° to 23°C for 2 hours. Various coating concentrations were used to determine optimal conditions. Coated microtiter plates were then washed 3 times with PBS solution containing 0.05% Tween, and remaining protein-binding sites were blocked by the addition of 0.02M Tris-buffered saline (0.9% NaCl) solution (pH, 7.4) containing 0.2% albumin. Collagen-coated microtiter plates were stored at 4°C for up to 30 days. Before use, plates were washed 3 times with PBS solution containing 0.05% Tween, loaded with standards as well as controls and test plasma samples, and processed for analysis of vWF:Ag, as described elsewhere.33 Calculation of ratios of vWF:Ag to vWF:CBA required that assays for vWF activity and vWF:Ag be performed in tandem. A monoclonal antibody to canine vWF was used to measure binding of vWF by collagen; this antibody replaced the polyclonal goat anti-canine vWF antibody used in the vWF:Ag assay. Plasma vWF:Ag concentration and activity were reported as a percentage of the vWF:Ag or vWF:CBA in a standard created from pooled plasma samples of 20 healthy dogs; the standard had an assigned value of 100% for vWF:Ag and vWF activity. The same lot of standard pooled plasma was used for all experiments. Samples were assayed in triplicate and results reported as the mean of the 3 determinations. Absorbance values were measured at 490 nm by use of a microplate reader. Data transformation and curve fitting were performed by use of software.

### Precision of the ELISA

Intra-assay precision was determined by triplicate analysis of 24 canine plasma samples with various concentrations of vWF that were assayed on the same microtiter plate. To establish interassay precision, aliquots of pooled canine plasma were prepared and stored frozen at −30°C until assayed. An aliquot was thawed to prepare serial plasma dilutions that had vWF:Ag concentrations ranging from 3.12% to 100%. Microtiter plates representing 9 plate coating lots were used; coated plates were stored at 2° to 8°C for up to 30 days prior to use.

### vWF cleaving protease

Canine plasma vWF was proteolytically degraded by ADAMTS13 essentially as reported for human vWF.20 Briefly, 0.5 mL of citrated canine plasma was added to dialysis cassettes with molecular-weight cutoff limits of 10,000 kDa. The cassettes were immersed at 37°C in a 30-fold excess volume of Tris-HCl (3 mM [pH, 8.3]; containing 1.5M urea) and dialyzed for various amounts of time (0 to 24 hours). Proteolysis was terminated by dilution with ELISA dilution buffer. The vWF:Ag and residual vWF:CBA were measured. Aliquots were reserved for analysis of vWF multimers.

### Analysis of vWF multimers

Analysis of vWF multimers was performed as described elsewhere on clinical samples that had discordant results for vWF:Ag and vWF activity. Analysis was also performed on urea-treated canine plasma samples to confirm ADAMTS13 proteolysis of vWF.

### Statistical analysis

Plasma samples were assigned to groups by use of defined limits for vWF:Ag concentration, the ratio of vWF:Ag to vWF:CBA, and breed (ie, Doberman Pinscher). Frequency distribution for all breeds in the population was determined. Descriptive statistics for groups were compiled; distributions were skewed. The Wilcoxon rank sum test (2-sided) was used to compare medians for vWF:Ag, vWF:CBA, and the ratio of vWF:Ag to vWF:CBA relative to hypothesized dichotomous risk factors (sex, bleeding history, status as type 1 vWD [vWF:Ag value > 0 but ≤ 49], or Doberman Pinscher with type 1 VWD). Three Kruskal-Wallis tests were used for nonparametric 1-way ANOVAs to simultaneously compare all 6 vWD groups (excluding type 3 VWD) with regard to vWF:Ag, vWF:CBA, and the ratio of vWF:Ag to vWF:CBA. Statistical calculations were performed by use of a commercial software package. Values of P < 0.05 (2 sided) were considered significant.
Results

Test development and optimization—When immobilized to polystyrene microtiter plate wells at a pH of 5.3, 7.4, or 9.6, bovine collagens from all sources bound canine vWF with maximal vWF binding at a pH of 9.6 (data not shown). Alkaline-immobilized bovine collagen supported generation of vWF concentration-dependent curves in dilutions of pooled canine plasma obtained from clinically normal dogs (Figure 1). At a coating concentration of 25 µg/well, type I collagen extracted by use of mildly acidic conditions and mixed collagen (types I and III) bound similar amounts of canine vWF. At low vWF concentrations (reciprocal logarithmic plasma dilution, ≤ 0.4), mixed collagen (types I and III) had higher absorbance values and was slightly more efficient as a binding substrate for vWF. The amount of canine vWF bound by type I collagen decreased when it was extracted by use of the moderately acidic conditions reported for extraction of collagen from fresh canine tendons. The other mixed collagen (types I and III) was least effective at this coating concentration for binding canine vWF.

When the coating concentration of bovine collagen was increased to 75 µg/well, mixed types I and III bovine collagen generated vWF concentration curves with desirable steep upward linear slopes in response to serial dilutions of pooled canine plasma from clinically normal dogs (Figure 2). Type I collagen extracted by use of moderately acidic conditions bound less canine vWF than the mixed types I and III commercial preparations and generated a shallower upward linear slope. Data for type I collagen extracted under mildly acidic conditions were not obtained because the yield of extracted collagen was too low to achieve the desired coating concentration of 75 µg/well.

Specificity of the absorbance signal for canine vWF was evaluated by use of pooled vWF-deficient plasma (< 0.01% vWF:Ag) obtained from dogs with type 3 vWD (Figure 3). Throughout the dilution range, the absorbance values at 490 nM for vWF-deficient plasma were ≤ 0.08 absorbance units. In contrast, the absorbance signal associated with comparable dilutions of normal pooled canine plasma (100% vWF:Ag) increased linearly throughout the dilution range and reached values > 1.0 absorbance units.

On the basis of performance characteristics, mixed bovine collagen (types I and III) was selected as the collagen source for the collagen-binding ELISA. It was used at a coating concentration of 75 µg/well in subsequent experiments.

Selectivity of mixed bovine collagen types I and III for preferential binding of high–molecular-weight canine vWF was evaluated (Table 1). Untreated pooled canine plasma from clinically normal dogs had comparable amounts of vWF:Ag and vWF:CBA. In contrast, when the pooled canine plasma from clinically normal dogs was depleted of high–molecular-weight forms of canine vWF by cryoprecipitate fractionation or activation of vWF protease for ≥ 1 hour, there was a pronounced reduction in vWF:CBA. Multimer analysis confirmed degradation of vWF and depletion of high–molecular-weight forms after protease treatment (Figure 4).

Ability of the collagen-binding ELISA to detect a reduction or total lack of high–molecular-weight forms of canine vWF was further evaluated by use of plasma samples obtained from dogs with inherited type 2 vWD (Table 2). Consistent with a reduction in high–molecular-weight forms, absorbance values at 490 nm for vWF-deficient plasma were ≤ 0.08 absorbance units. In contrast, the absorbance signal associated with comparable dilutions of normal pooled canine plasma (100% vWF:Ag) increased linearly throughout the dilution range and reached values > 1.0 absorbance units.
ular-weight multimers in type 2 vWD, vWF:Ag and vWF:CBA concentrations were discordant (median vWF:Ag concentration was 3.7 fold-higher than median vWF:CBA concentration). The median ratio of vWF:Ag to vWF:CBA for canine type 2 vWD was >2.0, which is similar to the ratio reported for humans with type 2 vWD and dogs with reduced amounts of high–molecular-weight multimers. Plasma samples from dogs with type 3 vWD had undetectable amounts of vWF:Ag and vWF:CBA.

Intra-assay and interassay precision were evaluated for the vWF:CBA. Mean intra-assay CV for 24 canine plasma samples that had vWF:Ag concentrations ranging from 1% to 148% and that were assayed in triplicate on the same microtiter plate was <5%. Interassay precision was established by use of serial dilutions of canine plasma assayed on 14 separate days. Mean vWF:CBA values for the dilutions were 101.2%, 49.7%, 24.9%, 12.3%, 6.6%, and 3.3%, respectively; and CVs were 1.2%, 3.9%, 3.8%, 3.8%, 8.7%, and 11.0%, respectively. When pooled canine plasma from clinically normal dogs was subjected to 4 freeze-thaw cycles, the recovered vWF:CBA after the fourth freeze-thaw cycle was 96% of initial collagen binding.

**Sample population**—Median age of dogs in the study population was 2.2 years, with a sex distribution of 126 (43%) females and 167 (57%) males. Reports of bleeding were included in the medical history of 35 (12%) dogs. A significantly (P = 0.01) higher percentage of males (64%) than females (36%) had bleeding reported as part of the medical history.

Purebred dogs represented 199 (68%) dogs of the population, whereas 85 (29%) dogs had no breed identification and 9 (3%) dogs were identified as mixed-breed dogs. Purebred dogs were distributed among 43 breeds spanning the major breed groupings. Breeds represented at highest frequency in the purebred sample population were Doberman Pinscher (70 [35%]), German Shepherd Dog (18 [9%]), Labrador Retriever (10 [5%]), Bernese Mountain Dog (10 [5%]), and German Wirehaired Pointer (10 [5%]). Solely on the basis of the vWF:Ag concentration, the majority of dogs (110 dogs representing Akita, American Pit Bull Terrier, Australian Cattle Dog, Bearded Collie, Bernese Mountain Dog, Bichon Frise, Borzoi, Boston Terrier, Boxer, Chesapeake Bay Retriever, Chihuahua, Chow Chow, Dachshund, Doberman Pinscher, German Shepherd Dog, German Shorthaired Pointer, German Wirehaired Pointer, Golden Retriever, Gordon Setter, Greyhound, Italian Greyhound, Irish Setter, Labrador Retriever, Leonberger, Mastiff, Miniature Pinscher, Newfoundland, Papillon, Pomeranian, Poodle, Rottweiler, Schnauzer, Shetland Sheepdog, Shiba Inu, Walker Hound, Weimaraner, Welsh Corgi, West Highland Terrier, and Whippet) had adequate amounts of vWF protein (vWF:Ag ≥50%). Ninety-two dogs representing 14 breeds (Australian Shepherd, Bernese Mountain Dog, Cocker Spaniel, Dachshund, Dalmatian, Doberman Pinscher, Fila Brasileiro, German Shepherd Dog, Greyhound, Jack Russell Terrier, Keeshond, Labrador Retriever, Poodle, and Shetland Sheepdog) were classified as deficient for vWF (vWF:Ag <50%). None of the dogs in the sample population had type 3 vWD.

**Screening for type 2 vWD**—The vWF variables used to establish vWD status of dogs screened in the study were summarized (Table 3). The concentration of vWF:Ag and clinical history were used for initial vWD classification. For the 293 dogs screened, 141 were classified as free of vWD. Median vWF:Ag for clinically normal dogs was 99%, whereas median vWF:CBA was slightly lower at 90%. Median ratio of vWF:Ag to vWF:CBA for dogs classified as clinically normal was 1.10.

### Table 1—Mean ± SD preferential binding of high–molecular-weight canine vWF to a commercial preparation of mixed (types I and III) bovine collagen.

<table>
<thead>
<tr>
<th>Plasma treatment</th>
<th>Activation time (h)</th>
<th>VWF:Ag (%)</th>
<th>vWF activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0</td>
<td>102.0 ± 0.8</td>
<td>97.0 ± 1.3</td>
</tr>
<tr>
<td>Cryoprecipitate removal</td>
<td>0</td>
<td>9.0 ± 0.2</td>
<td>3.0 ± 0.2</td>
</tr>
<tr>
<td>vWF protease activation</td>
<td>1.0</td>
<td>84.0 ± 3.2</td>
<td>34.0 ± 1.3</td>
</tr>
<tr>
<td></td>
<td>2.0</td>
<td>86.0 ± 5.8</td>
<td>5.0 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>24.0</td>
<td>89.0 ± 3.4</td>
<td>0.3 ± 0.1</td>
</tr>
</tbody>
</table>

*Serial dilutions of pooled plasma from clinically normal dogs (assigned a value of 100% for vWF:Ag and activity) were used to generate dose-dependent calibration curves. Coefficients of variation ranged from 1.3% to 7.6%.

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Figure 3—Dose-response curves obtained for pooled canine plasma (vWF:Ag concentration, 100%) obtained from clinically normal dogs (squares) and vWF-deficient canine plasma (vWF:Ag concentration, 0.01%) obtained from dogs with type 3 vWD (circles) immobilized by use of mixed (types I and III) bovine collagen (75 µg/well). Equivalent serial dilutions were used.
Twenty-seven dogs in the sample population had antigen values between 50% and 69% and were classified as indeterminate status. The vWF:Ag and vWF:CBA concentrations for these dogs were similar. Median ratio of vWF:Ag to vWF:CBA was 1.12 and not significantly different from that for dogs classified as free of vWD or having type 1 vWD, excluding Doberman Pinschers.

Dogs with vWF:Ag values > 0 and ≤ 49% were classified as presumed type 1 vWD. This group consisted of 119 dogs whose median vWF:Ag concentration was 19% and represented 43% of the samples screened. Median vWF:CBA concentration for all breeds classified as type 1 vWD was concordant with median vWF:Ag to vWF:CBA was 0.94. Doberman Pinschers with type 1vWD had a significantly (vWF:CBA was 0.94. Doberman Pinschers with type 1 vWD was concordant with median vWF:Ag to vWF:CBA was 0.94. Doberman Pinschers with type 1 vWD had a significantly (P < 0.001) lower ratio of vWF:Ag to vWF:CBA than other breeds with type 1 vWD or any other group, except dogs with type 3 vWD. This finding indicates higher concentrations of circulating, more functional vWF.

Six dogs with a median vWF:Ag concentration of 12% and median vWF:Ag concentration 4-fold higher than the vWF:CBA concentration were classified as having type 2 vWD. Multimer analysis confirmed the lack of high–molecular-weight vWF in the plasma from type 2 vWD dogs (Figure 5). When samples were resubmitted for confirmation, discordant results for the ratio of vWF:Ag to vWF:CBA could not be duplicated. Therefore, the original submissions were assigned a classification of acquired type II vWD rather than inherited type 2 vWD. It was not possible by use of medical histories or follow-up monitoring to establish a clinical disease status. It is therefore possible that the observed reduction in vWF:CBA concentration may reflect in vitro changes rather than changes secondary to disease.

None of the dogs in the sample population had inherited type 2 vWD. Thus, the frequency of type 2 vWD in this study did not approach the 8% reported in another study in which values for vWF:CBA were used as a screening test. None of the dogs in the sample population were identified with type 3 vWD.

**Discussion**

Evaluation of vWF activity is used routinely for the laboratory diagnosis and classification of type 2 vWD in humans. This contrasts with vWD in dogs in which vWF activity is rarely evaluated. The RCoF assay is most commonly used to evaluate vWF function in humans and measures vWF-dependent agglutination of platelets induced by the antimicrobial ristocetin. The poor performance characteristics of the RCoF assay when used with canine plasma have limited its use in diagnosis of vWD in dogs. The snake venom botrocetin can replace ristocetin and improves the measurement of vWF function in mammalian plasmas and has been used to monitor vWF activity in humans and dogs. An alternate procedure gaining widespread acceptance as superior to the RCoF and botrocetin assays is the measurement of vWF:CBA. Collagen-binding assays are more sensitive, more reproducible, and easier to standardize than other functional assays. A collagen-binding assay requiring cumbersome and nonstandard extraction of collagen from freshly dissected canine gastrocnemius tendon has been used to diagnose vWD in dogs and evaluate changes in vWF activity after desmopressin administration.

In the study reported here, we described a collagen-binding ELISA that uses commercially available bovine collagen as a standard vWF binding substrate to enhance standardization and overcome the need for

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**Table 2**—Median (range) values for vWF:Ag, vWF:CBA, and the ratio of vWF:Ag to vWF:CBA for dogs with adequate vWF and abnormal control dogs with inherited type 2 or type 3 vWD.

<table>
<thead>
<tr>
<th>Status</th>
<th>No. of dogs</th>
<th>vWF:Ag (%)</th>
<th>vWF:CBA (%)</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adequate vWF1</td>
<td>141</td>
<td>99 (70–167)</td>
<td>90.0 (46.0–172.0)</td>
<td>1.1 (0.5–1.7)</td>
</tr>
<tr>
<td>Type 2T</td>
<td>4</td>
<td>2.8 (1.5–4.8)</td>
<td>0.7 (0.3–1.4)</td>
<td>3.9 (3.4–5.8)</td>
</tr>
<tr>
<td>Type 3</td>
<td>8</td>
<td>&lt; 0.01</td>
<td>&lt; 0.05</td>
<td>NC</td>
</tr>
</tbody>
</table>

* Plasma vWF:Ag and vWF:CBA are reported as the percentages of values determined for pooled control plasma from clinically normal dogs. Plasma samples had vWF:Ag concentration > 50%.

1 On the basis of ratios for vWF:Ag to vWF:CBA ≥ 2.0 and confirmed lack of or severe reduction in high–molecular-weight vWF multimers (≤ 6,000 kd), as determined on the basis of analysis of multimers by use of SDS–1% agarose gels. The lower limit of detection was 0.8% for vWF:Ag and 0.05% for vWF:CBA.

NC = Not calculated.
burdensome collagen extraction. The ELISA was configured to facilitate tandem measurement of vWF:CBA and vWF:Ag by use of the same buffers (coating, washing, sample dilution, and development), reagents, and antibodies for the vWF:Ag assay used in the laboratory of the authors. The collagen-binding ELISA was then used to screen for type 2 vWD in plasma samples from 293 dogs whose vWD classification was assigned on the basis of vWF:Ag concentration alone.

The possibility that bovine collagen could bind canine vWF was suggested by another study conducted by our laboratory group in which it was revealed that canine platelets adhered to immobilized bovine collagen and there was successful use of mixed types I and III collagen from bovine or equine sources in human collagen-binding assays. The performance characteristics of the canine collagen–binding assay when used with mixed types I and III bovine collagen, including its lower limit of detection and ability to discriminate between quantitative (type 1 vWD) and qualitative (type 2 vWD) defects for dogs with vWD, are similar to collagen-binding assays optimized for humans with vWD.

The ratio of vWF:Ag to vWF:CBA for clinically normal humans and those with type 1 vWD is close to 1. Clinically normal dogs and dogs with type 1 vWD in the study reported here also had concordant ratios of vWF:Ag to vWF:CBA with a value close to 1, which is in agreement with ratios obtained by use of species-specific collagen.

In contrast to other breeds with type 1 vWD, we observed a slight increase in vWF activity relative to vWF:Ag in Doberman Pinschers with type 1 vWD and a significant (P < 0.001) decrease in the mean ratio of vWF:Ag to vWF:CBA. The lower ratio is consistent with increased amounts of the more active high–molecular-weight forms of vWF in the plasma of Doberman Pinschers.

The ratio of vWF:Ag to vWF:CBA for clinically normal humans and those with type 1 vWD is close to 1. In our sample population, 6 of 293 dogs had discordant values between vWF:Ag and vWF:CBA with the ratio of vWF:Ag to vWF:CBA exceeding 2. Multimer analysis confirmed the lack or severe reduction of high–molecular-weight vWF; therefore, these 6 dogs were classified as having a type 2-like defect. We attempted to determine whether the dogs had an inherited form of type 2 vWD through follow-up testing of resubmitted samples to confirm persistently abnormal results for the collagen-binding assay. Testing of resubmitted samples for 3 dogs failed to confirm inherited type 2 vWD; abnormal results for

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Table 3—Classification for vWD and median (range) values for vWF variables for 293 plasma samples obtained from dogs.

<table>
<thead>
<tr>
<th>Classification</th>
<th>No. of dogs</th>
<th>vWF:Ag (%)*</th>
<th>vWF:CBA (%)*</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Free of vWD†</td>
<td>141</td>
<td>99 (70–167)</td>
<td>90.0 (46.0–172.0)</td>
<td>1.1 (0.5–1.7)</td>
</tr>
<tr>
<td>Indeterminate for vWD</td>
<td>27</td>
<td>60 (50–69)</td>
<td>52.0 (31.0–75.0)</td>
<td>1.1 (0.8–1.9)</td>
</tr>
<tr>
<td>Type 1 vWD</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All breeds</td>
<td>119</td>
<td>19 (2–48)</td>
<td>19.0 (2.0–57.0)</td>
<td>0.9 (0.3–2.0)</td>
</tr>
<tr>
<td>Doberman Pinscher</td>
<td>58</td>
<td>10 (2–48)</td>
<td>14.0 (3.0–57.0)</td>
<td>0.8 (0.3–1.8)</td>
</tr>
<tr>
<td>All breeds except Doberman Pinscher</td>
<td>61</td>
<td>27 (3–48)</td>
<td>22.0 (2.0–48.0)</td>
<td>1.09 (0.5–2.0)</td>
</tr>
<tr>
<td>Type 2 vWD‡</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inherited</td>
<td>0</td>
<td>ND</td>
<td>ND</td>
<td>NC</td>
</tr>
<tr>
<td>Acquired</td>
<td>6</td>
<td>12 (1–43)</td>
<td>3.0 (0.2–15.0)</td>
<td>5.2 (2.0–16.5)</td>
</tr>
<tr>
<td>Type 3 vWD</td>
<td>0</td>
<td>ND</td>
<td>ND</td>
<td>NC</td>
</tr>
</tbody>
</table>

ND = Not detected.

See Table 2 for remainder of key.
the collagen-binding assay were transitory and consistent with acquired type 2 vWD. Samples for resubmission could not be obtained for the other 3 dogs (1 dog had died, and the other 2 dogs were unavailable). In the 3-year period following the study reported here, only 3 dogs (2 German Shorthaired Pointers and 1 mixed-breed dog) were identified with inherited type 2 vWD, in contrast to identification of 22 dogs with type 3 vWD.

In another study, 28% of dogs with vWD were identified with type 1 vWD and 4% with type 3 vWD. Inclusion of vWF activity in the diagnostic criteria changed the classification for 7 of 21 (33%) dogs between type 1 and type 2 vWD. Investigators in that study did not distinguish between inherited or acquired forms of type 2 vWD. On the basis of an abnormal vWF:Ag concentration, 43% of dogs classified with type 1 vWD and 0% with type 3 vWD. When results for the collagen-binding assay were included, 6 of 125 (5%) dogs tentatively classified with type 1 vWD were reclassified as type 2 vWD.

A large national registry of 1,286 humans with vWD had a distribution of 73% type 1, 21% type 2, and 6% type 3 vWD. In a referral-based study that used a cutoff limit of < 50% for vWF:Ag concentration as the diagnostic criterion for vWD, the distribution in humans was 87% for type 1, 13% for type 2, and 0% for type 3 vWD. The referral-based study reported here also used a < 50% cutoff value and had a distribution of 93% type 1, 5% type 2, and 0% type 3 vWD. In the canine population screened in another study, 58% of dogs with vWD were classified with type 1, 29% with type 2, and 13% with type 3. Limitations for use of referral-based prevalence estimates that have been discussed for humans with vWD also apply for dogs with vWD, including over- or underreporting related to inclusion and sampling biases.

In light of the high incidence of inherited type 2 vWD in dogs reported in another study as well as no prior reports of acquired vWD in dogs, it was surprising to us that 5% of the dogs identified as deficient for vWF in the study reported here had an acquired type 2-like defect. In human medicine, pathogenic mechanisms proposed to explain type 2-like defects seen in association with underlying diseases include lymphoproliferative, myeloproliferative, neoplastic, immunologic, and cardiovascular disorders.

Abnormal clearance of large vWF multimers can result from formation of antibodies to vWF and removal of the immune complexes, adsorption onto tumor cells or activated platelets, proteolytic degradation, and mechanical degradation of vWF by high shear stress. Investigators in another study reported a reduction in plasma vWF concentration and the relative lack of high–molecular-weight vWF in Cavalier King Charles Spaniels with myxomatous mitral valve disease and moderate-to-severe mitral regurgitation. Those authors attributed the changes to mechanical degradation of vWF. The collagen-binding assay may be of use for screening dogs to detect this phenomenon.

We could not establish the underlying disease or diseases associated with the acquired type 2-like defects identified in the study reported here. Prospective studies designed to identify diseases associated with changes in vWF activity should be conducted to discern the pathophysiologic processes of acquired type 2 vWD in dogs. We cannot rule out possible in vitro changes for vWF as a result of improper sample collection or handling. However, rejection criteria for vWF:CBA analysis were identical to those used for analysis of vWF:Ag, including rejection of specimens when fibrin formation was evident.

We conclude that mixed types 1 and 3 bovine collagen from a readily standardized commercial source can be used to configure an ELISA to measure canine vWF:CBA. The collagen-binding ELISA is sensitive to depletion of high–molecular-weight vWF multimers in canine plasma and discriminated between types 1 and 2 vWD. Inherited type 2 vWD can be detected in dogs at a frequency lower than that predicted by estimates for type 2 vWD in humans and inferred from another study in dogs.

In dogs, inherited type 2 vWD may be as rare as type 3 vWD. In the 3-year period after the study reported here, 3 dogs (2 German Shorthaired Pointers and 1 mixed-breed dog) of approximately 10,000 tested were identified with inherited type 2 vWD, in contrast to identification of 22 dogs with type 3 vWD. Finally, acquired type 2 vWD should be considered in dogs with clinical signs similar to those for dogs with congenital vWD and a predisposing underlying disease.

References


