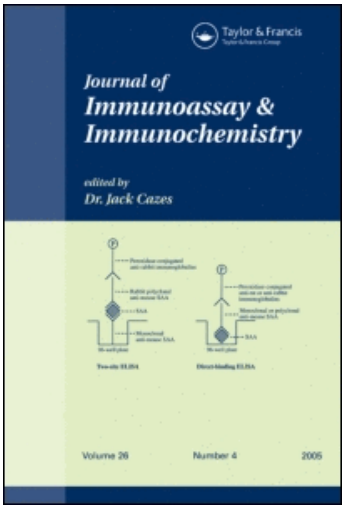

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A Sensitive Immunoassay for von Willebrand Factor

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A SENSITIVE IMMUNOASSAY FOR VON WILLEBRAND FACTOR

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ABSTRACT

We have developed an ELISA specific for canine von Willebrand factor antigen (vWF:Ag) that also strongly reacts with the VWF:Ag of humans and many other vertebrates. This assay was designed to avoid the use of immunoreagents of human origin, however, commercially available antibodies to human vWF:Ag may also be used. von Willebrand factor (vWF) was quantitated using a modified double-sandwich ELISA with polyclonal antibodies specific for canine vWF:Ag. The assay was as sensitive for measuring canine vWF:Ag as previously published immuno-radiometric assays and the most sensitive ELISA for human vWF:Ag. Employing commercially available antibodies to human vWF:Ag in the same double-sandwich configuration, the lower limit of detection for human vWF:Ag was 4.8×10^{-6} units/ml, lower by a factor of ten than previously reported ELISAs. In addition, a wide range of vWF:Ag levels can be determined with just a single plasma dilution. The assay readily distinguishes type III von Willebrand disease from other types of von Willebrand disease having very low levels of vWF. This vWF ELISA can be used to evaluate large numbers of plasma samples simultaneously and is therefore well-suited for large-scale screening programs.

Key words: (von Willebrand factor, ELISA, Human, Canine)

INTRODUCTION

von Willebrand factor (vWF) is a large platelet-adhesive glycoprotein required for normal hemostatic plug formation(1-2). vWF also serves as a protective carrier protein for coagulation factor VIII(3-4). Qualitatively distinct pools of vWF are present in

endothelial cells, platelets, and plasma(5). In normal plasma, vWF circulates as a series of multimers with molecular weights ranging from 0.5 to greater than 15 million daltons(6). The largest vWF polymers appear to exhibit the greatest hemostatic activity(7).

Quantitative or qualitative changes in vWF result in von Willebrand's disease (vWD), which is the most common inherited bleeding disorder in man(8) and domestic animals(9). Recent estimates of the gene frequency of inherited, congenital vWD in humans range from 1 in 500 to as high as 1 in 50 live births of either sex(8), whereas in select inbred dog families prevalence can be as high as 70%(9).

The clinical manifestations and laboratory diagnostic features of vWD are very heterogeneous. Within the three major classes of vWD at least 20 subtypes have been described(10). Diagnosis, treatment, and management of this heterogeneous defect requires accurate measurement of plasma and platelet vWF.

The antigenic component of vWF can be quantitated by reaction with specific antibodies in immunoassays and is termed von Willebrand factor antigen (vWF:Ag). Several enzyme-linked immunosorbent assay (ELISA) methods for vWF:Ag have been reported(11-14), which have safety, efficiency, and sensitivity advantages when compared to the Laurell electroimmunoassay (EIA)(15-16) and immuno-radiometric assay (IRMA)(17).

We undertook the development of an ELISA capable of strongly reacting with both human canine and other vertebrate vWF:Ags for use in our ongoing comparative studies of human and animal vWD. Since immunological reagents used in previously reported ELISAs for vWF have been prepared using human plasmas as the source of immunogen and may have the potential of transmitting blood-borne diseases to other humans, a secondary objective of this study was to develop an ELISA with comparable sensitivity to previously described ELISAs that would avoid this disadvantage. Data

derived from the use of this assay in other species are the subject of another publication(18).

MATERIALS AND METHODS

Plasmas:

Platelet-poor plasma (PPP) was obtained from canine blood and stored as previously described(16). A standard plasma pool was prepared by mixing equal volumes of PPP from ten hemostatically normal, healthy dogs (five of each sex) and equated to 1.00 unit/ml (100%) vWF:Ag. Three individual reference plasmas (normal, type I, and type III vWD) were stored in multiple small aliquots at -50°C. These reference plasmas were obtained from a colony of dogs with hemostatic defects maintained and characterized at the Comparative Hematology Laboratory of the New York State Department of Health at Albany, New York(19). Plasma from three previously diagnosed type III vWD Shetland sheepdogs(20) and three type III Scottish Terriers(21) were also utilized. Additional citrated canine PPP samples utilized in this study were shipped frozen to our laboratory as part of our vWD screening program and stored at -20°C until assayed. Human standard (equated to 1.0 unit/ml by comparison to reference plasma purchased from Helena Laboratories, Beaumont, TX, USA) and vWF:Ag-depleted plasmas were purchased from BioData Corporation (Horsham, PA, USA) in lyophilized form and reconstituted according to the manufacturer's instructions.

Production of Antibodies to Canine vWF:Ag:

Two separate batches of canine vWF:Ag were partially purified from plasma by cryoprecipitation and passed over 2% and 6% agarose columns as previously described(16). Antibodies to vWF:Ag were prepared in rabbits and rendered monospecific by absorption with cryoprecipitate of plasma from a Scottish terrier with

type III vWD(21). A polled Alpine goat was immunized with the second batch of vWF:Ag using a similar protocol as the rabbits but with doubling of the volume of the primary and booster immunizations. After eight weekly injections, 400 ml of the goat blood was collected from the jugular vein into glass test tubes and permitted to clot at 37°C for one hour and then overnight at 4°C. The serum was de-complemented by heating to 56°C for 30 minutes and depleted of residual coagulation factors with tricalcium phosphate and absorbed twice with type III canine vWD cryoprecipitate. Globulin and IgG were prepared from the goat and rabbit antisera using standard techniques, dialyzed versus phosphate buffered saline (PBS), and stored at -50°C.

Antibodies to Human vWF:Ag:

Goat anti-human factor VIII related antigen - IgG fraction (lot #55797) was purchased from Atlantic Antibodies, Scarborough, ME. Rabbit immunoglobulin to human vWF (#A082) was purchased from Dako Corporation, Santa Barbara, CA.

ELISA reagents:

Coating buffer: 0.05M sodium carbonate, pH 9.6. Blocking buffer: 0.9% NaCl, 0.2% bovine serum albumin (BSA) (Sigma, St. Louis, MO, USA, #A6793) 0.003M Tris free base, pH 7.4. Dilution buffer: 0.9% NaCl, 0.05% BSA, 0.003M Na EDTA, 0.02M Tris free base, pH 7.4. Citrate buffer: 0.033 M citric acid, 0.067M Na_2HPO_4, pH 5.0. Washing buffer: phosphate buffered saline, 0.5% V/V Tween-20, pH 7.4 (PBS-Tween). All of the above buffers contained 0.1 mg% thimerosal. Indicator substrate: 30 mg tablet O-phenylenediamine dihydrochloride (OPD) (Sigma, St. Louis, MO, #P8412) dissolved immediately before use in 30 ml of citrate buffer plus 150 ul of 3% H_2O_2.

Plate Coating and Titration:

The rabbit anticanine or antihuman vWF IgG was diluted with coating buffer and 100 ul

was dispensed per well of Immulon II microtiter plates (Dynatech, Alexandria, VA, USA). The plates were covered and incubated overnight in a moist 37°C chamber. The microplate wells were then washed three times with PBS-Tween; 200 ul of blocking buffer was added per well. The plate wells were sealed and stored overnight at 4°C prior to use. The plate wells remained immunologically reactive for at least one month when stored in this manner.

To establish working canine and human plasma and antibody concentrations, checkerboard titrations of the various antibody and antigen reagents were utilized to establish those combinations of reagent dilutions which generated, after a 10-min OPD reaction time, an absorbance for the blank close to zero yet not exceeding 1.5 for the most concentrated plasma vWF:Ag standard. 100-ul plasma dilutions were incubated overnight in wells at 4°C (a one-hour room- temperature incubation may also be used). The goat (sandwich) and pig antigoat IgG-peroxidase conjugated (Tago, Inc., Burlingame, CA, USA) antibodies were subsequently added for one hour incubations each at room temperature. Between the plasma and antibody additions, the plate wells were washed three times with 100 ul of the PBS-Tween, which was removed by shaking. Following the pig antibody steps and washing of wells, the plate was sprayed three times with citrate buffer. A 100-ul aliquot of the substrate solution was added to each well. The horseradish peroxidase (HRP) reaction and OPD color change were stopped after approximately 10 minutes with 100 ul/well of 5N H_2SO_4.

Once the optimum antibody concentrations were established, buffer blanks and serial canine and human plasma dilutions from 1:25 to 1:409,600 were tested in their respective antibody systems in quadruplicate to determine linear regions of the dose response curve and lower limits of sensitivity. The dose response for human plasma was also determined in the canine antibody system. The globulin fraction of the sandwich antibody against canine vWF:Ag was reacted against canine plasma dilutions

while the IgG form was reacted against the human vWF:Ag dilutions. Purified reagents have been found to increase the sensitivity of heterologous antibody-antigen systems. The absorbance at 492 nm was determined in an EL312 Microplate Reader (Bio-Tek, Winooski, VT, USA). The Bio-Tek KinetiCalc software program was used for data transformation and curve fitting.

Precision:

Intra- and inter-assay precision for canine vWF:Ag was determined by repeatedly assaying the same plasma dilution on the same microtiter plate or measuring the vWF:Ag level of aliquots of the same plasma on different plates over a period of several weeks.

Sample Storage and Refreezing:

Individual canine plasmas or their dilutions were stored at 4°C or -20°C for four days and thawed (as necessary) and assayed daily for vWF:Ag.

Routine Canine vWF:Ag Assay:

Plates were coated with rabbit anticanine vWF:Ag IgG at 1:500 as described and following washing and addition of blocking buffer were sealed with tape and stored at 4°C. Three or four serial plasma dilutions and a buffer blank were used to construct a standard curve for vWF:Ag with the 1:200 dilution used as the most concentrated standard. Test plasmas were assayed at a 1:500 or 1:600 dilution. Plasma dilutions were prepared and added to the plate wells in the afternoon, incubated at 4°C overnight, and dilutions of goat globulin at 1:250 and pig antigoat IgG at 1:10,000 were added consecutively for 1-hour incubations at room temperature. The washing and substrate reaction steps were as described above.

Correlation between Laurell EIA and ELISA vWF:Ag Assays:

Frozen canine plasma samples (590 total) shipped to our laboratory for clinical vWD screening were thawed and assayed for vWF:Ag simultaneously by the EIA technique(16) and this ELISA.

Statistical Comparisons:

Statistical analysis were performed using an NCSS software package (Dr. J. Hintz, Kaysville, UT, USA). The Wilcoxon test for matched pairs (2-tailed) was used to compare mean vWF:Ag levels. P values less than 0.01 were considered significant.

RESULTS

Preliminary results with the modified double-sandwich ELISA for canine vWF:Ag demonstrated that plates coated with the capture antibody at dilutions up to 1:4000 immobilized plasma vWF:Ag. Titration of the coating antibody to canine vWF:Ag using a constant dilution of standard pooled plasma (1:100), goat (sandwich) and pig (conjugate) antibodies indicated that capture of the vWF:Ag did not significantly increase below the 1:250 dilution. Similarly the sandwich antibody did not bind additional vWF:Ag below the 1:250 dilution. It was not possible to achieve saturation using the HRP-conjugated pig anti-goat IgG with a one-hour incubation with dilutions as concentrated as 1:250. A 1:10,000 dilution of the conjugate antibody was selected because, in combination with the above capture and sandwich antibody dilutions, an absorbance of approximately 1.5 was generated for the 1:100 dilution of the standard pooled plasma after 10 minutes reaction time.

Using the above saturating antibody concentrations for the rabbit and goat anti-canine vWF:Ag antibodies, serial dilutions of the standard canine plasma from 1:25 to 1:409,600 produced the concentration response shown in Fig. 1. Data from six

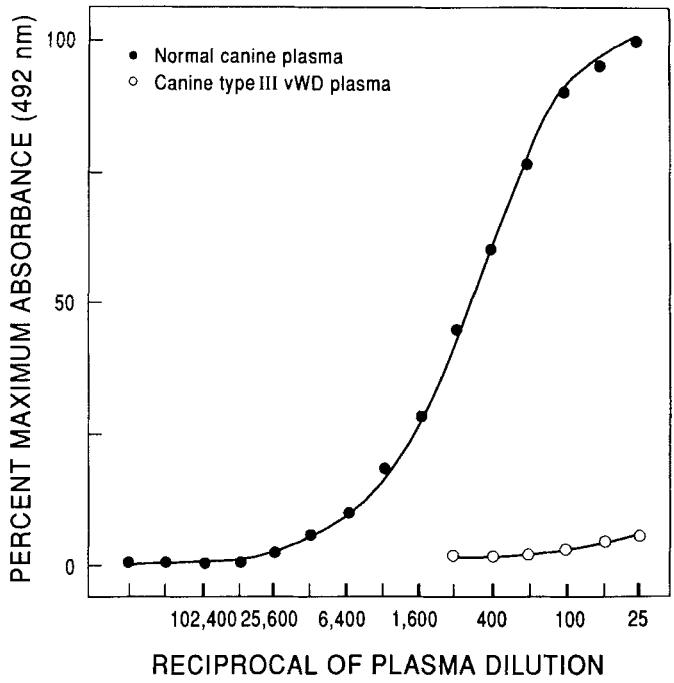

Figure 1. Canine von Willebrand factor antigen (vWF:Ag) quantitated by species-specific enzyme-linked immunosorbent assay (ELISA). Dose-response relationship for normal pooled plasma (●) and from plasma from a dog with type III von Willebrand's disease (vWD) (○).

experiments demonstrated that, at plasma concentrations of 1:25,600 and greater, the optical densities were higher than the average buffer blank plus four standard deviations. When plasma from three type III vWD Scottish terriers(21) and three type III Shetland sheepdogs(20) were serially diluted 1:25 to 1:800 and assayed in triplicate in each of the above six experiments, only the most concentrated dilutions were reactive above the buffer blank.

Similar dose-response experiments were performed with the human antibody system and dilutions of human plasma (Figure 2A). Commercial antihuman vWF:Ag

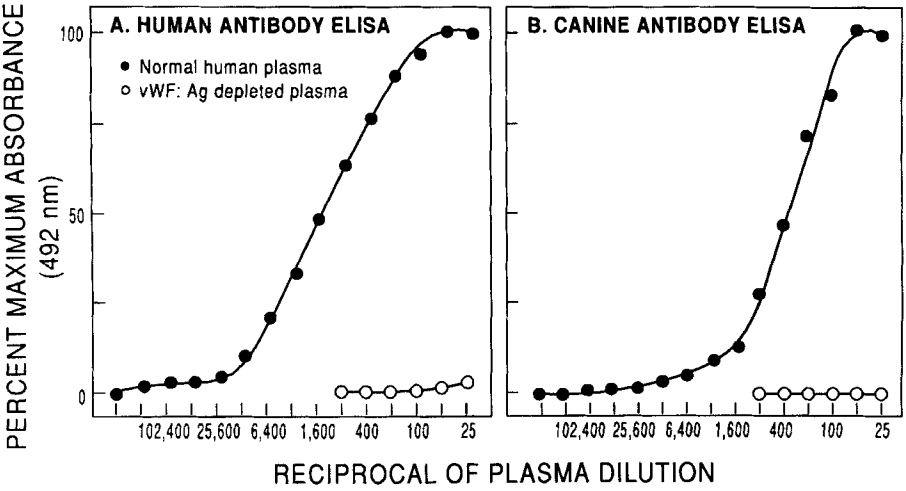

Figure 2. Human vWF:Ag quantitated by ELISA using human or canine specific capture and sandwich antibodies. Normal reference plasma (•) and vWF:Ag depleted plasma (o). Panel A - antibodies specific for human vWF:Ag. Panel B - antibodies specific for canine vWF:Ag.

antibody dilutions used for the coating and sandwich antibodies were 1:4000 and 1:1000, respectively. The human plasma dilution with the lowest vWF:Ag concentration that generated absorbances significantly higher than the blank was 1:204,600. For data presented in Figure 2A, the blanks (n=12) had a mean absorbance value of 0.002 $\pm$ 0.006. The 1:409,600, 1:204,800, and 1:102,400 dilutions had mean absorbances of 0.004, 0.027, and 0.048, respectively. The human vWF:Ag-depleted plasma generated absorbances slightly above the blanks at the three most concentrated dilutions.

Dose-response-curve experiments were also conducted with standard human plasma using the canine specific capture and sandwich antibodies. The results are shown in Figure 2B and demonstrate absorbances parallel to those observed with the specific human reagents; however, the vWF:Ag in the depleted plasma was

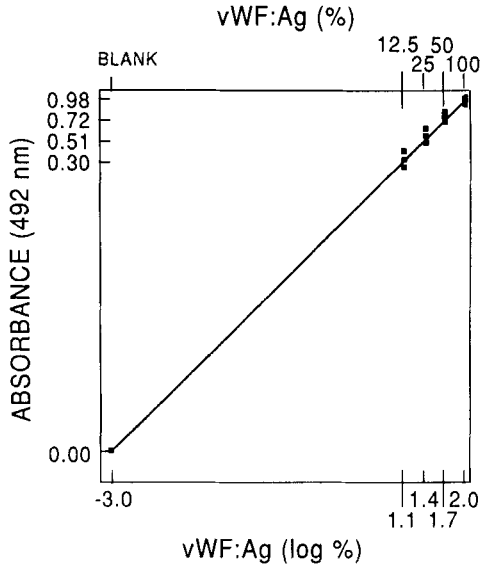

Figure 3. Standard curve for normal pooled canine plasma employing a logarithmic transformation and quadratic curve fit.

undetectable. The lower limit of detection for human plasma in the canine ELISA system was 1.56×10^{-4} units/ml.

Serial dilutions of the standard canine plasma ranging from 1:200 and 1:1600 were used to establish standard curves for the canine specific capture/ sandwich antibody system. The 1:200 standard plasma dilution was equated to 100% vWF:Ag. Absorbance data were then analyzed using eight curve-fitting options available with the KineticCalc software package. The best fits were obtained using linear or quadratic curve fitting with logarithmic transformation of both absorbance and percent vWF:Ag values. As shown in Fig. 3, the dose-response curves for standard normal canine plasma were linear for a wide range of vWF:Ag concentrations when the quadratic option was used.

Dose parallelism was evaluated for 34 clinical canine plasma samples with levels of vWF:Ag ranging from 6 to 145%. Samples were assayed in triplicate at three serial dilutions. When the quadratic and linear curve-fitting methods were compared for the data set, we found statistically insignificant differences between the mean vWF concentrations at the 1:300 and 1:600 dilution for those plasmas with $\geq$ 30% vWF:Ag (n=22). Dose response parallelism was also maintained for the low ($\leq$ 15%) vWF:Ag plasmas (n = 12) at the 1:75 and 1:150 dilutions; the differences were not statistically significant.

The linear fit failed to discriminate type III vWD reference (vWF:Ag < 0.002%) and test plasmas from those with less than 10% vWF:Ag. vWF antigen values reported for the type III reference ranged from 1 to 10%. In contrast, when type III vWD plasmas were assayed in parallel with 72 clinical canine plasma samples (single assay dilution at 1:600; triplicate wells) having a range of vWF:Ag from 5 to 150%, the quadratic curve fit yielded comparable results to the linear method throughout the range of vWF:Ag values and consistently identified type III vWD plasmas.

The internal precision of this ELISA was evaluated for three canine plasmas with known vWF:Ag levels (75, 60, and 45%). Quadruplicate dilutions (1:600) were prepared for each plasma and each was then assayed in triplicate on the same microtiter plate. External assay precision was evaluated by using separate frozen aliquots of the three above plasmas on four successive days performed by different ELISA operators. The average coefficient of variation (CV) for the triplicate determinations of each sample dilutions was 7.89% (n=48). The average CV of each of the separate, identical dilutions repeated in the same assay (interdilutional variation) was 6.34%. The average CV of these 12 dilutions in four different assays (interassay precision) was 7.8%. When assayed multiple times on separate days, three reference plasmas with mean vWF:Ag levels of 86, 64% and 7.6% had CV's of 13.2% (n=21), 7.1% (n=18), and 29.2% (n=20) respectively.

TABLE I

Effect of Sample Incubation Time on Canine Plasma vWD:Ag Level Assayed in a Species Specific ELISA.

	Length of Sample Incubation Before Plate Development			
	Plasma vWF:Ag(%)			
Sample #	16 hr	40 hr	64 hr	CV
1	80	90	71	11.8
2	52	51	52	1.1
3	152	106	110	8.8
4	89	70	91	13.9
5	77	64	86	14.6
6	128	115	135	8.0
7	89	111	96	11.3
8	99	86	100	8.2
9	42	45	48	6.7
10	91	106	109	9.5
11	184	200	166	9.3
12	102	98	90	6.3
13	76	81	70	7.3
14	297	248	250	10.4
15	54	66	51	13.9
16	78	90	70	12.7
17	5	7	4	28.6
18	16	23	16	22.0
19	6	9	8	19.9
20	3	4	4	15.7
21	5	8	5	28.8
22	10	12	11	9.1
Mean	77.6	76.8	74.7	12.6

The effect of repeated freeze-thaw cycles on canine plasma vWF:Ag was evaluated by dispensing four different freshly prepared plasmas in 0.5-ml aliquots and freezing them at -20°C. Three aliquots of each plasma were then thawed at 37°C and refrozen from one to three additional times. All four freeze-thaw aliquots for each sample were thawed and assayed simultaneously and in triplicate at the 1:600 dilution. The experiment was repeated three times with separate freeze-thaw aliquots of the same

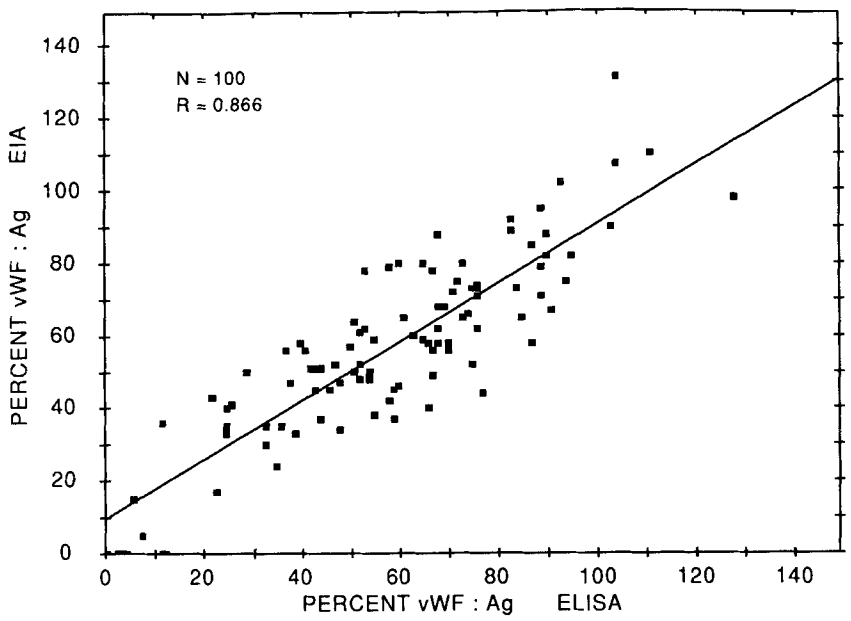

Figure 4. Scatter plot diagram comparing plasma vWF:Ag levels measured by EIA and ELISA. Regression analysis: $y=9.53 + 0.81x$, $r=0.866$. $S_y=57.7 \pm 24.3$, $S_x=59.3 \pm 25.8$.

starting plasma. No significant changes in vWF:Ag levels through the second, third, and fourth thaw cycles were observed.

The effect of prolonged sample incubation with microtiter plate-immobilized capture antibody was evaluated to establish whether the antigen incubation step might be performed over the weekend (Table 1). Twenty-two clinical samples were diluted and loaded in an identical manner on three separate microtiter plates with appropriate standards and references. Each plate was developed with the sandwich, indicator antibodies and substrate after 16, 40, or 64 hours of storage at 4°C. The results demonstrated that sample incubation at 4°C for up to three days did not significantly influence the quantitation of plasma vWF:Ag. Mean vWF:Ag for 16, 40, and 64 hours for

the 22 samples were 77.6 $\pm$ 68.3, 76.8 $\pm$ 61.1, 74.7 $\pm$ 60.6; respectively. The average CV for the 22 samples determined three times was 12.6%.

Once assay conditions were optimized we then examined the correlation between this vWF ELISA and the Laurell EIA routinely utilized in our laboratory to measure vWF:Ag. The scatter plot (Fig. 4) demonstrates overall agreement between assays. The correlation coefficient between methods for 100 test plasmas was 0.866. When n was expanded to 590 test samples the correlation coefficient between assays for the larger sample population was 0.85. However, disparate assay results (> 50% difference between assay values) were consistently obtained for a small cohort of animals. These samples represent less than 7% of the total 590 samples tested. The reason(s) for the observed differences of this small subgroup remain to be established.

DISCUSSION

This study was undertaken to develop an ELISA for canine vWF:Ag with a sensitivity high enough to permit measurement of previously undetectable vWF:Ag levels in canine cellular and platelet lysates and vWD plasmas. The extremely sensitive nature of this canine assay prompted an evaluation of a similarly constructed ELISA specific for human vWF:Ag, which demonstrated a higher level of sensitivity for the detection of human vWF:Ag than previously reported. We have also found that canine-specific antibodies can be used to quantitate vWF:Ag in human plasma.

Specific polyclonal antibodies to canine vWF:Ag were prepared and purified by salting out and ion-exchange chromatography. Once saturating antibody conditions were established, a dose response curve demonstrated that normal canine plasma could be diluted 25,000-fold and consistently generate absorbances higher than the blank. The lower limit of vWF:Ag detection in this assay system is at least 4×10^{-5} units vWF/ml or approximately 2,000 times more sensitive than the 9.0×10^{-2} units vWF/ml of the

canine EIA(16). Furthermore the lower limit of detection for human vWF:Ag is decreased to 4.8 x 10^{-6} units/ml when commercially available antibodies to human vWF:Ag are used. The lower limit of detection for canine vWF:Ag using homologous reagents is similar to that published for a human vWF:Ag-specific ELISA using a biotin/avidin indicator system(22). When commercially available reagents are used in the modified double-sandwich ELISA configuration of our assay the sensitivity for human vWF:Ag increases by a factor of ten over the most sensitive vWF ELISA published to date(22).

A modified double-sandwich ELISA for human vWF:Ag with a minimum limit of detection of 1.0 X 10^{-3} units/ml has been previously described(23). Silveira et al.(24) reported an ELISA for human vWF:Ag with a lower limit of detection of 3.0 X 10^{-4} units/ml and attributed the increased sensitivity to the use of monomeric Fab-HRP conjugates employed for antigen detection. Furlong et al.(25) published data for a vWF:Ag ELISA with a sensitivity of 2.0 x 10^{-4} units/ml. Their assay included a single 2-hour co-incubation of antigen and monoclonal conjugate in polyclonal coated microtiter wells. When viewed together these studies suggest that highly purified immunological reagents, a prolonged antigen incubation, and a modified sandwich configuration contribute to increased ELISA sensitivity for vWF:Ag.

The ability of the canine antibody system to generate a specific and linear dose response for human vWF:Ag indicates the immunological similarity of canine and human vWF:Ag and suggests the utility of these canine reagents for the quantitation of human vWF:Ag. We have also observed strong cross-reactivity of the canine antibody system with other mammalian vWF:Ag; this observation is the subject of a separate report(18).

The linearity of the dose-response curve for canine vWF between 1:200 to 1:1600 dilutions of standard plasma was exploited to develop a quantitative ELISA for canine vWF:Ag. Six previously diagnosed type III canine vWD plasmas were evaluated at serial dilutions from 1:25 to 1:800, and similar to previous reports for some(22,25), but not

all(26) human type III vWD plasmas, generated absorbances above background at the 1:25 and 1:50 dilutions but not at the 1:400 and 1:800 dilutions. Human vWD type III plasma was not evaluated, however vWF:Ag-immunodepleted human plasma behaved in an identical fashion to the canine type III plasmas. The finding of significant absorbances at the 1:25,600 and 1:204,600 dilutions of normal canine and human plasmas respectively, suggests that the reactive material in the human vWF-immunodepleted and vWD type III canine plasmas at the 1:25 dilutions may have been caused by non-specific protein binding. Alternatively, the sandwich antibodies may lack monospecificity or the canine type III plasmas have extremely low, but detectable amounts of vWF:Ag.

A review of eight different curve fitting systems available on the software package was undertaken to optimize data transformation so that plasmas with trace amounts (less than 1%) vWF:Ag could be identified. Incorporation of a zero standard and quadratic curve fitting allowed accurate quantitation of trace vWF:Ag. Dose-response studies demonstrated that this curve fit yielded quantitative data for plasma vWF:Ag throughout the range of plasma dilutions. Accurate quantitation of vWF:Ag levels less than 15% required plasma dilution at 1:75, in contrast to all others, which could be quantitated using the 1:600 dilution. However, even at the 1:600 dilution, the assay clearly discriminated low-level vWF:Ag samples from type III vWD plasmas.

The precision of this canine assay compared favorably with the data published for the Laurell EIA utilized in this laboratory. The CV by ELISA for 12 samples repeated in four different assays was 7.8% compared with an average of 8.2% for six samples repeated in seven different EIA's. Others have reported average interassay imprecision values of 12.8%(14) for EIA and 3.8-13.3% for ELISA(13,22).

It has been the experience of this laboratory that it may be necessary to reassay a sample for vWF:Ag and therefore the effect of repeated freeze-thawing was

investigated. After three freeze-thaw cycles, the vWF:Ag level of four plasmas remained unchanged.

The effect of longer canine plasma incubation periods in microplate wells was evaluated to determine if vWF:Ag ELISA's could be started and completed several days apart to accommodate staff scheduling needs and still maintain a uniform flow of results in a large-scale vWF:Ag testing program. Our data demonstrate that addition of 24 to 48 hours to the standard 16-hour, 4°C incubation period had no statistically demonstrable impact on the results. The 22 samples tested had virtually identical vWF:Ag levels regardless of whether the plates were developed after one, two, or three days of sample incubation.

Finally, we compared the vWF:Ag levels of 590 clinical samples by this ELISA and our laboratory's routine Laurell EIA. An overall correlation coefficient of 0.85 between assays was observed, which is very similar to results reported by other laboratories assaying human vWF(25,27,28). Correlations greater than 0.90 have also been reported(12,22,26,29,30). However, with the exception of Mazurier et al.(12), who reported an r value of 0.91 (n=167), the number of samples in each comparison was less than 100 (n=20-85). Reproducible yet clearly disparate results were obtained for select plasmas, suggesting that the two assays may identify different epitopes on the vWF protein. This is reinforced by our observation that the sandwich reagent (goat anti-vWF:Ag) is a weak precipitating antibody in the EIA(16). Other physical factors could also be responsible for the disparate results.

The advantages of ELISA over EIA including improved efficiency and dramatically increased sensitivity for vWF:Ag advance this technique's applicability in clinical, diagnostic (human and veterinary), and biomedical research laboratories. The enhanced sensitivity of the ELISA described in this study is likely related to a combination of prolonged sample incubation in the capture step and the utilization of a second indicator antibody in preference to a conjugated sandwich reagent.

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