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Research Article

The Relationship Between von Willebrand Factor Gene and von Willebrand Factor Antigen Levels in Dogs [1]

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Abstract

In this study, the relationship between plasma vWF antigen level and polymorphism occurring due to the leucine (L) to proline (P) substitution at position 2380 in exon 42 in von Willebrand factor gene (*VWF*) in dogs was aimed to investigate. The present study was performed on 161 dogs of various breeds and ages (95 male, 66 female) referred to Faculty of Veterinary Clinics at Erciyes University between January 2014 and 2015. Blood samples were collected in EDTA and Na citrate tubes after clinical examination of the dogs. PT, APTT, TT, fibrinogen, D-dimer, thrombocyte, vWF antigen (vWF: Ag) levels and *VWF* were determined from the blood samples. Genotypes were examined using PCR and restriction endonucleas enzymes. In the laboratory examination, 34 (21.1%) of the cases were positive and 127 (78.9%) of the cases were negative with concern to von Willebrand. Prevalence of the disease in different dog breeds that included to the present study were Golden Retreiver (n=35) 34.3%, Kangal (n=45) 11.1%, German Shepherd (n=11) 36.4%, Labrador Retriever (n=7) 42.9%, English Cocker Spaniel (n=2) 100%, English Pointer (n=8) 25.0%, mix breed (n=8) 33.3%, Husky (n=7) 12.5%, Malinois (n=1) 100%, Dogo Argentino (n=2) 50.0%, Samoyed (n=1) 100% and some other breeds were 0%. The genotype of *VWF* was not statistically significant in both positive and negative dogs with concern to vWF: Ag values (P=0.675). When comparison were made in terms of proportional distribution in positive and negative dogs, statistical importance were not observed between genotype and the disease ratio (P=0.969). In conclusion, relationship between *VWF* and vWF antigen level were not determined in dogs. According to our knowledge; this study is the first repot on *VWF* and vWF: Ag relationship therefore believed to be important.

Keywords: *Coagulation, Dog, Haemostasis, Mutation, von Willebrand factor*

Köpeklerde von Willebrand Faktör Antijen Seviyesi ve von Willebrand Faktör Geni Arasındaki İlişkinin Belirlenmesi

Özet

Bu çalışmada, köpeklerde von Willebrand faktör geninin 42. ekzonunun 2380. pozisyonunda meydana gelen lösin (L) - prolin (P)'nin yer değiştirmesine neden olan mutasyon sonucu oluşan polimorfizm ile plazma vWF antijen seviyeleri arasındaki ilişkinin araştırılması amaçlandı. Çalışmaya, Ocak 2014 ve 2015 yılları arasında, Erciyes Üniversitesi Veteriner Fakültesi Kliniklerine getirilen farklı ırk ve yaşta (95 erkek, 66 dişi) 161 köpek dahil edildi. Köpeklerin klinik muayeneleri yapılarak EDTA ve Na sitratlı tüplere kan örnekleri toplandı. Kan örneklerinde PT, APTT, TT, fibrinojen, D-dimer, trombosit, vWF antigen (vWF: Ag) seviyeleri belirlendi ve *VWF* elde edildi. Genotipler, yapılan PCR sonucunda elde edilen ürünlerin *Msp*I endonükleaz enzimi ile kesilerek belirlendi. Laboratuar muayenesinde 34 köpek (%21.1) von Willebrand açısından pozitif olarak belirlenirken, 127 köpeğin (%78.9) negatif olduğu belirlendi. Çalışmaya dahil edilen hayvanlarda ırklara göre hastalığın görülme oranları Golden Retreiver (n=35) %34.3, Kangal (n=45) %11.1, German Shepherd (n=11) %36.4, Labrador Retriever (n=7) %42.9, English Cocker Spaniel (n=2) %100, English Pointer (n=8) %25.0, melez (n=8) %33.3, Husky (n=7) %12.5, Malinois (n=1) %100, Dogo Argentino (n=2) %50.0, Samoyed (n=1) %100 ve diğer ırklar %0 olarak belirlendi. Hem pozitif hem de negatif köpeklerde *VWF* genotipi ile vWF: Ag değerleri arasında istatistiksel olarak bir ilişkili bulunamadı (P=0.675). vWD pozitif ve negatif köpeklerde oransal dağılım açısından karşılaştırıldığında hastalık oranı ve genotip arasında istatistik bir önem gözlenmedi (P=0.969). Sonuç olarak, köpeklerde vWF antigen seviyesi ve *VWF* arasında ilişki bulunmadı. Bilgilerimize göre bu çalışma, *VWF* and vWF: Ag arasındaki ilişkinin araştırıldığı ilk çalışma olması nedeniyle önemlidir.

Anahtar sözcükler: *Pıhtılaşma, Köpek, Hemostaz, Mutasyon, von Willebrand faktör*

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INTRODUCTION

Von Willebrand's disease (vWD) is the commonest an acquired or inherited bleeding disorders of dogs and humans being [1-3].

The most inherited haemostatic disorder of dog is von Willebrand's disease. Von Willebrand syndrome is transferred as autosomal recessive character in dogs [4]. The quantitative deficiencies or qualitative abnormalities of the von Willebrand's factor (vWF) or both causes bleeding tendency at the patients suffers from vWD [1,3]. The vWD phenotype can be classified as type I, II, and III on the basis of the plasma vWF concentration and the vWF multimer structure [1,2]. The measurement of plasma vWF antigen (vWF: Ag) concentration is the current gold standard for diagnosis of the vWD and have been used as screening test for carrier detection in dogs [2]. It is reported that vWD is negative in dogs with vWF: Ag concentration >70% and is positive in dogs with vWF: Ag concentration <50% [2,5]. At the nucleotide sequences of von Willebrand factor gene (*VWF*) is determined to be 85% identical of human and canine and can be used as cross-species modelling of their vWDs [3]. The human *VWF* is located near the telomere of the short arm of chromosome 12 and split into 52 exons, spanning 180 kb of deoxyribonucleic acid [3].

In dogs; *VWF* has 178 kb size, and is in dog caryotype on number 12 chromosome, which has multiple repeated regions [6]. Two widespread mutations about inherited presence of von Willebrand syndrome have been determined. One of the mutations is on the number 4 exon and the other is on the number 42 exon [7].

Venta and Vidal [8] found a polymorphism of the canine *VWF* which can be characterized using the *Msp*I restriction enzyme. This polymorphism occurs due to the leucine (L) to proline (P) substitution at position 2380 in exon 42 in the canine *VWF* [8].

We aimed to investigate the relationship between plasma vWF antigen level in different breed dogs and polymorphism occurring due to the L to P substitution at position 2380 in exon 42 in *VWF* in dogs. Furthermore, we proposed to determine whether the different simultaneous distribution with the vWD phenotype and vWF: Ag concentration as a criteria of elimination in the future for selecting vWD free dogs.

MATERIAL and METHODS

In the present study, 161 dogs which vaccinated, not pregnant, out of eustrus cycles and have no hypothyroidism signs of various breeds and ages (95 male, 66 female) referred to Faculty of Veterinary Clinics at Erciyes University between January 2014 and 2015 were used as animal material. Blood samples were collected into EDTA and Na citrate tubes after clinical examination of the dogs. The citrated blood was centrifuged at 1.500 x g for 15 min at room temperature, and then plasma was separated. Complete blood counts were examined by electronic cell counter (Mindray BC-2800 Vet®, China). All plasma and blood samples with EDTA were stored at -80°C until needed.

Von Willebrand's factor antigen were performed on a CS-5100 analyser (Sysmex UK Ltd, Milton Keynes, UK), all reagents were from Siemens Healthcare Diagnostics. Prothrombin time (PT), Activated thromboplastin time (APTT), thrombin time (TT), fibrinogen, D-dimer were analysed on the Sysmex CA-7000 (Siemens Healthcare Diagnostics) at the Central Laboratory of Erciyes University.

The DNAs were extracted using phenol-chloroform method from leukocytes. PCR analysis for the gene was performed in a 25 µl reaction mixture, containing 1.5 mM $MgCl_2$, 50 µM of dNTP mix, 0.2 µM of each primers, 1 X PCR buffer, 1U Taq polymerase and 50 ng of genomic DNA template. The *VWF* was amplified using the primers: primer 1 5'-TCTTGTCCCCCGCACTGGA-3'; primer 2 5'-TGGTTGTGG TGCAGCCACAGTC-3'. Primers were designed to the canine *VWF* sequence (Genbank accession numbers L76227, U66246, AF099154) by Venta and Vidal [8]. Thermal cycling conditions were followed by 50 cycles of 94°C 30 sec, 62°C 1 min and 72°C 1 min and PCR products were digested at least 60 min at 37°C with the 10U *Msp*I restriction endonuclease (MBI Fermentase®, Lithuania).

The Chi-Square Test ($\chi 2$) was used to check whether the populations were in Hardy-Weinberg equilibrium. Genotypic data were compared to vWD prevalence by using Chi-Square Test in MİNİTAB 16 software.

Erciyes University Local Board of Ethics Committee for Animal Experiments has approved the study protocol of this research (Decision no: 2013/09).

RESULTS

Dogs included to the study were 95 male (59%), 66 female (41%). A comparison of vWF: Ag concentration between males and females were found no significant difference (P>0.05). Out of 161 animals 75 (46.6%) were < 1 years old and 86 (53.4%) were >1 years old. There were no statistically significant effects of age on vWF: Ag levels (P>0.05). According to vWF: Ag concentration; higher than 50% of this concentration (127, which was about 78.9%) considered negative and lower than 50% of vWF: Ag level (34, which was about 21.1%) considered positive [2]. In positive breeds according to vWF: Ag concentration; allele frequencies the *VWF* and prevalence of vWD given in *Table 1*.

The PCR amplification was yielded a 147 bp product. The PCR products were digested with 10U of *Msp*I restriction endonuclease. Restriction digestion of 147 bp PCR products with *Msp*I enzymes revealed three genotypes *(Fig. 1)* of LL

Table 1. *The allele frequencies and prevalence the VWF in vWD positive dog breeds according to vWF: Ag concentration*
Tablo 1. *vWF: Ag seviyelerine göre vWD pozitif olan köpek ırkların VWF genotip frekansları ve vWD görülme oranları*

Breed	N (%)		Genotypes		
	vWD Negative	vWD Positive	LL	LP	PP
Golden Retriever	23 (65.7%)	12 (34.3%)	5	6	1
Kangal	40 (88.9%)	5 (11.1%)	4	1	0
German Shepherd	7 (63.6%)	4 (36.4%)	1	1	2
Labrador Retriever	4 (57.1%)	3 (42.9%)	1	1	1
English Cocker Spaniel	0 (0%)	2 (100%)	1	0	1
English Pointer	6 (75.0%)	2 (25.0%)	1	1	0
Mixed breed	4 (66.7%)	2 (33.3%)	1	0	1
Husky	7 (87.5%)	1 (12.5%)	0	1	0
Malinois	0 (0%)	1 (100%)	1	0	0
Dogo Argentino	1 (50.0%)	1 (50.0%)	0	0	1
Samoyed	0 (0%)	1 (100%)	0	0	1
Other Breeds	35 (100%)	0 (0%)			

N: *Sample Size;* ***vWD:*** *von Willebrand Disease*

Table 2. *The allele, genotype frequencies and Hardy-Weinberg chi-square analysis of the VWF*
Tablo 2. *VWF'nin allel, genotip frekansları ve Hardy-Weinberg Ki-kare analizi*

Groups	Genotypes Frequency			Allele Frequency		Statistical Significant (Chi-squared HWE)
	LL	LP	PP	L	P	
vWD Negative	53 (41.73%)	43 (33.86%)	31 (24.41%)	58.7	41.3	$X^2 = 11.57$, $P < 0.001$ (df = 1)
vWD Positive	15 (44.12%)	11 (32.35%)	8 (23.53%)	60.3	39.7	$X^2 = 3.576$, $P = 0.059$ (df = 1)

HWE: *Hardy-Weinberg Equilibrium;* ***X^2:*** *Chi-Square value; df: Degree of freedom;* ***vWD :*** *von Willebrand Disease;* ***VWF:*** *von Willebrand factor gene*

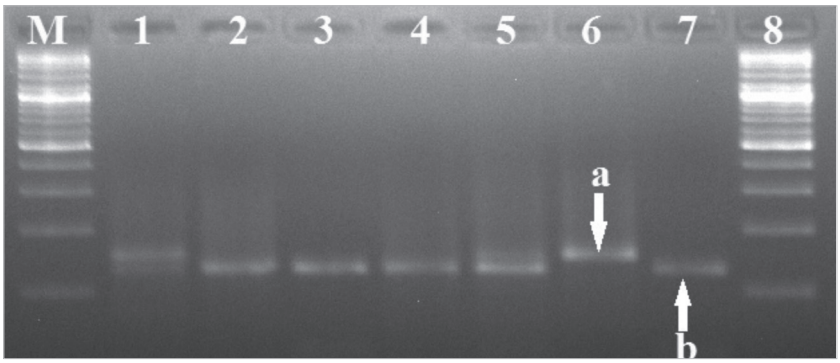

Fig 1. Electrophoresis of RFLP of canine *VWF* after digestion by *Msp*I. Lanes 2-4 and 7; PP (-/-) genotypes; Lanes 1 and 5: LP (+/-) genotypes; lane 6: LL (+/+) genotype; lane M: DNA ladder (100 bp); a: 147 bp and b: 124 bp

Şekil 1. Köpek *VWF* geninin *Msp*I enzim kesim ürünlerinin elektroforez görüntüsü. 2-5 ve 7 nolu kuyucuklar: PP (-/-) genotipli bireyler; 1 ve 5 nolu kuyucuklar: LP(+/-) genotipli bireyler; 6 nolu kuyucuk: LL(+/+) genotipli birey. M: 100 bç'lik DNA merdiveni; a: 147 bç ve b: 124 bç

(+/+) (147 bp), LP (+/-) (147 and 124 bp) and PP (-/-) (124 and 23 bp).

The allelic, genotypic frequencies and heterozygosity of the *VWF*, polymorphisms for the dog are given in *Table 2*.

There was no significant difference between *VWF* genotypes and vWF: Ag values in vWD positive and vWD negative dogs (P=0.675) *(Table 3)*.

In addition, no statistical significance was observed for the prevalence between vWD and genotype (P=0.969), compared the proportional distributions according to genotypes of the *VWF* in vWD positive and vWD negative dogs *(Table 4)*.

The differences between vWD positive and vWD negative dogs in levels of D-dimer, fibrinogen, leucocytes, erythrocyte, haemoglobin and PCV were significant (P<0.05) *(Table 5)*.

DISCUSSION

Von Willebrand's disease is the most common bleeding disease in humans and dogs, resulting various clinical

Table 3. *The proportional distributions of vWF: Ag concentration according to genotypes*
Tablo 3. *Genotipe göre vWF: Ag konsantrasyonunun oransal dağılımı*

Genotype	N	Mean ±SEM	Statistical Significance (ANOVA)
LL	68	66.16 ± 2.51	F: 0.394 P = 0.675
LP	54	68.67 ± 2.95	
PP	39	70.10 ± 4.61	

***vWD:** von Willebrand Disease; **vWF:** Ag: von Willebrand factor antigen; **N:** Sample size; **SEM:** Standard error of the mean; **F:** F statistics value; **P:** P value*

Table 4. *The proportional distributions according to genotypes of the VWF in vWD positive and vWD negative dogs*
Tablo 4. *vWD pozitif ve negatif köpeklerde VWF'nin genotipine göre oransal dağılımı*

Genotype		vWF: Ag		Total
		-	+	
LL	Count	53	15	68
	%	77.9%	22.1%	100.0%
LP	Count	43	11	54
	%	79.6%	20.4%	100.0%
PP	Count	31	8	39
	%	79.5%	20.5%	100.0%
Total	Count	127	34	161
	%	78.9%	21.1%	100.0%

***vWF:** Ag: von Willebrand factor antigen; **vWD :** von Willebrand Disease*

Table 5. *Some hematological parameters in vWD positive (1) and vWD negative (0) dogs*
Tablo 5. *vWD pozitif (1) ve negatif (0) köpeklerde bazı hematolojik parametreler*

Parameters	vWD	N	Mean± SEM	Statistical Significance (Student T test)
D-dimer (µg/l)	0	127	2914.96±611.49	T = 2.91 P = 0.04
	1	34	1063.82±174.69	
Prothrombin time (sec)	0	127	7.30±0.08	T = 0.380 P = 0.780
	1	34	7.24±0.10	
Activated thromboplastin time (sec)	0	127	17.09±0.19	T = 0.887 P = 0.376
	1	34	16.74±0.25	
Thrombin time (sec)	0	127	17.75±0.19	T = 0.732 P = 0.466
	1	34	17.44±0.42	
Fibrinogen (mg/dl)	0	127	294.06±12.90	T =0 .491 P < 0.001
	1	34	193.03±16.06	
Thrombocyte (10^3/µl)	0	127	313.42±12.18	T = 1.788 P = 0.077
	1	34	281.97±12.69	
Leucocyte (10^3/µl)	0	127	20.72±1.06	T = 2.138 P = 0.035
	1	34	17.32±1.19	
Erythrocyte (10^6/µl)	0	127	6.28±0.14	T = -2.544 P = 0.012
	1	34	7.03±0.22	
Haemoglobin (g/dl)	0	127	15.54±0.30	T = -2.578 P = 0.011
	1	34	17.18±0.53	
Haematocrit (%)	0	126	44.55±0.87	T = -2.582 P = 0.011
	1	34	49.47±1.75	

***vWD:** von Willebrand Disease; **N:** Sample size; **SEM:** Standard error of the mean; **T:** T statistics value; **P:** P value*

conditions from none to death causing severe hemorrhagic diathesis [9,10]. In vWD; to determine diagnosis and carriers, vWF: Ag levels have been used. According to Brooks et al.[11] and Kayar et al.[12] vWF: Ag concentrations classified as non-carrier if it is 70-179%, borderline if it is 51-59%, carrier if it is 2-49% and effected if it is 0.1-0.3% in individuals. On the other hand, Raymond et al.[13] defined over 60% vWF: Ag concentration as non-carrier, between 50-59% as border-line, and between 0-49% as carriers. In addition, Pathak [14] evaluated 70-180% as normal range, 50-69% as borderline and 1-49% as carrier. In the present study; vWF: Ag concentration <50% individuals were considered to be vWD positive as reported by Burges et al.[2] and Thomas [15].

Von Willebrand's disease is an acquired or inherited bleeding disorder common in humans and dogs [3]. In human beings estimated prevalence of vWD has been reported between 0.01-0.02% [9,16]. Castaman et al.[17] point out that with a prevalence of 1% in general population; the ratio of visible clinical symptoms is only 0.01%.

The prevalence of vWD in Scottish Terrier dogs has been reported to change between 18% to 30% [18-21]. In German Wirehaired Pointers; carrier ratio reported to be 12% and disease developing ratio is reported to be 1% [11]. On the other hand, Dutch Kookier dogs this ratio determined to be 5.2% (in 717 dogs; 38 dogs had type III vWD) [22]. According to Brooks et al.[19] when lower than 50% vWF: Ag concentration taken into consideration; 73% of dobermans, 30% of scotties, and 28% of shelties had abnormal vWF: Ag concentration. Furthermore; Kayar et al.[12] found this ratio in German Shepherd dogs as 26%. In our study, when lower than 50% vWF: Ag concentration evaluated; vWD ratio was determined to be 21.1%. Prevalence of the disease in different dog breeds that included to the present study were Golden Retreiver (n=35) 34.3%, Kangal (n=45) 11.1%, German Shepherd (n=11) 36.4%, Labrador Retriever (n=7) 42.9%, English Cocker Spaniel (n=2) 100%, English Pointer (n=8) 25.0%, mixed breed (n=8) 33.3%, Husky (n=7) 12.5%, Malinois (n=1) 100%, Dogo Argentino (n=2) 50.0%, Samoyed (n=1) 100% and some other breeds were 0%. The difference observed in the prevalence; believed to be occurred due to diagnostic method, animal breed and number of animals used in studies.

Brooks et al.[1] examined German Wirehaired Pointer dogs and found no relation between vWD, age and sex. Similary, Kayar et al.[12] also found no relation between vWD and age. In the present study no correlation between the occurrence of the vWD, sex or age in dogs also determined.

In vWD dogs; number of platelet, TT, PT, APTT times were reported to be in generally reference values or APTT time were slightly a bit longer than normal [2,12,14,22]. Similarly, in the present study, platelet number, TT, PT and APTT times were in reference borders and differences between two groups were not significant.

In the present study, serum fibrinogen level in dogs with vWD were lower statistically (P<0.001) compared to the level obtained from the dogs without vWD but the determined concentrations in both groups were within the reference values. Obtaining normal fibrinogen levels in vWD dogs was in agreement with the previous studies [15]. D-dimer is a product of cross-linked of fibrin primer degradation [23]. Determining low D-dimer level in vWF: Ag level low dogs can be explained by the insufficient formation of clot which found in the present study.

It is important that the affected dogs and asymptomatic carriers of vWD should be excluded from breeding programs. Quantitative assays of plasma vWF antigen concentration have been used for definitive diagnosis of vWD and have been used to detect vWD carrier dogs [1,19,24,25]. Additionally, Brooks et al.[11] reported that a single vWF: Ag levels can be applied breeding dogs selection without vWD in German Wirehaired Pointers. However, Slappendel et al.[22] speculated that plasma vWF: Ag levels ranged between 30 to 89% in 39 Dutch kookier dogs are obligate carriers of Type III vWD. In addition, in people for the estimates of the heritability is used as plasma vWF: Ag levels in between 35-75% [16]. Because of the uncertainty in defining carrier status by ELISA method measuring vWF: Ag concentration in plasma, the location and character of the mutation causing vWD should be determined [3]. Identification of the affected gene and its action mechanism is also important in developing treatment for vWD. The encode of *VWF* is located on short arm of chromosome 12 and the 52 exons of the gene in humans [9,26]. The single-gene associations between plasma vWF: Ag concentrations and variants at the angiotensin-coverting enzyme, the FUR2 locus, lipoproteinreceptor-related protein, and arginine vasopressin 2 receptor have been reported in previous studies in humans [16]. Kramer et al.[3] investigated German Shorthaired Pointers having type II vWD and they found statistical importance between vWF variant nucleotide in exon 28 and vWF multimer deficiencies. Furthermore, in Scottish Terriers single base deletion in fourth exon were determined [18].

Venta and Vidal [8] reported that in exon 42 of the canine *VWF,* a polymorphic region cutting by an *Msp*I is present. In parallel with Venta and Vidal [8] findings; in the present study at 11 different dog breeds, *VWF* had polymorphic regions when the gene cut by an *Msp*I. Furthermore, breed specific difference in different dog breeds on the examined cutting regions were not determined in the present study as reported by Venta and Vidal [8].

In the present study, 11 different dog breeds raised in Turkey were investigated. In this study, apart from Venta and Vidal's [8] study, the relationship between *Msp*I restriction site polymorphism and vWF: Ag levels in plasma were examined. As a result of the present study; relationship between *Msp*I cutting region and vWF: Ag levels could not be determined. The reason could be; investigated each breed a vWD; the levels of vWF: Ag were different levels as reported by Slappendel et al.[22], or it was thought that the number of animals that diagnosed as vWD were too low. Furthermore, in the present study it was believed that, *Msp*I region that used genotyping of dog breeds could not be effective in determining individuals having mutant allele. To test these possibilities; dog breeds that the disease seen more often, studies having more animal materials should be performed in the future.

As a result of the present study; the relationship between plasma vWF antigen level and *Msp*I polymorphism at position 2380 in exon 42 in *VWF* in dogs could not be determined. In the present study; it was thought that the detection of *Msp*I polymorphism at position 2380 in exon 42 in *VWF* is not sufficient for the definitive diagnosis of vWD. In addition to this, to own best knowledge, the present study is the first report on *VWF* and vWF: Ag relationship, thus this study will contribute to the literature. Furthermore, the other importance of the present study may also come from, its' one of rare study with concern to clinical syndrome and their molecular relationship. Besides; vWF: Ag levels and vWD reported first time in Kangal breed dogs. Additionally; presence of *Msp*I polymorphism on the *VWF* determined first time in the Kangal breed dogs that raised in Turkey.

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