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A single nucleotide polymorphism in the canine cytochrome *b₅* reductase (*CYB5R3*) gene is associated with sulfonamide hypersensitivity and is overrepresented in Doberman Pinschers

Jennifer M. Reinhart, DVM, MS, DACVIM (SAIM), Joanne Ekena, MS, Amelia C. Cioffi, and Lauren A. Trepanier, DVM, PhD, DACVIM (SAIM), DACVCP

Department of Medical Sciences, School of Veterinary Medicine, University of Wisconsin-Madison, Madison, WI 53706

Abstract

Canine sulfonamide hypersensitivity (HS) has been associated with a variant in the cytochrome *b₅* reductase gene (*CYB5R3* 729A>G), which encodes a drug-detoxifying enzyme. Study objectives were to determine variant allele frequency in Doberman Pinschers (DOBE), a breed which may be predisposed to sulfonamide HS, and to characterize the effects of *CYB5R3* 729G on gene expression and function. *CYB5R3* 729A>G allele frequencies were compared between DOBE (n = 24) vs. non-Doberman (nonDOBE; n = 60) dogs. *CYB5R3* mRNA expression, protein expression, and reduction of sulfamethoxazole hydroxylamine were compared between banked canine liver samples of 729AA vs. GG genotype. The 729G allele was overrepresented in DOBE (1.00) vs. nonDOBE dogs (0.567, *p* < 0.0001). mRNA and protein expression as well as cyt *b₅* reductase activity were similar between livers of AA and GG genotype. All Doberman Pinschers in this study were homozygous for *CYB5R3* 729G, which could contribute to this breed's apparent predisposition to sulfonamide HS. However, *CYB5R3* 729G does not alter sulfamethoxazole detoxification capacity, so a direct role could not be demonstrated. It is possible that this marker is linked to another contributing variant.

Keywords

Genotype; Idiosyncratic; Potentiated sulfonamide; Adverse Drug Reaction

Introduction

Hypersensitivity (HS) to sulfonamide antibiotics is an uncommon, but potentially severe, idiosyncratic drug reaction that occurs in both dogs and humans (Trepanier, 2004). In dogs,

Correspondence to: Lauren A. Trepanier.

DR JENNIFER M REINHART (Orcid ID : 0000-0002-3021-5075)

Conflict of Interest Statement

The authors have no conflicts of interest to report.

Authorship Contributions Statement

All authors were involved in the conception and design of the study (JR, JE, LT), acquisition of data (JR, AC), analysis and interpretation of data (JR, AC, LT), or drafting and revision of the manuscript (JR, LT). All authors have read and approved the final manuscript.

sulfonamide HS occurs a minimum of 5 days and an average of 12 days after initiation of antimicrobial therapy (Trepanier et al., 2003). The most commonly reported clinical signs include fever, blood dyscrasias, hepatopathy, and polyarthropathy, but uveitis, cutaneous and mucocutaneous lesions, proteinuria, and neurologic signs may also occur (Fox et al., 1993; Giger et al., 1985; Harvey, 2006; Trepanier et al., 2003). Treatment is largely supportive and requires withdrawal of the sulfonamide drug; our previous retrospective study found a mortality rate of 21%, with hepatopathy and thrombocytopenia carrying a poorer prognosis (Trepanier et al., 2003).

As an idiosyncratic drug reaction, the occurrence of sulfonamide HS is inherently difficult to predict. However, in humans, the condition appears to be heritable (Mohanasundaram, Rajaram, & Mohanasundaram, 1998; Neuman, Malkiewicz, & Shear, 2000; Shear et al., 1986) and, in dogs, breeds such as the Doberman Pinscher appear to be overrepresented (Cribb, 1989; Cribb & Spielberg, 1990; Giger et al., 1985; Gray, 1990; Werner & Bright, 1983; Whur, 1987). These findings suggest that a genetic predisposition to sulfonamide HS may be present for both species.

Our laboratory has previously identified a genetic risk factor for sulfonamide HS in dogs: a single nucleotide polymorphism (SNP) in the *CYB5R3* gene (729A>G) (Funk-Keenan et al., 2012). *CYB5R3* encodes for cytochrome *b₅* reductase, a hepatic microsomal enzyme that detoxifies the sulfonamide hydroxylamine drug metabolite, which is thought to be involved in the pathogenesis of the HS reaction (Cribb & Spielberg, 1990). In our previous study, the GG genotype was significantly overrepresented in dogs with sulfonamide HS compared to dogs tolerant to a course of sulfonamide antibiotics. The *CYB5R3* 729A>G variant is a synonymous coding SNP, but is predicted to alter the conformation of the mRNA transcript to a more stable form (Funk-Keenan et al., 2012). We therefore hypothesized that *CYB5R3* 729G might lead to impaired mRNA translation, decreased enzyme synthesis, and diminished sulfonamide hydroxylamine reduction activity.

The goal of this study was to better understand the role of *CYB5R3* 729A>G as a genetic risk factor for sulfonamide HS in dogs. The specific objectives of this study were to determine whether *CYB5R3* 729G is overrepresented in Doberman Pinschers and to evaluate the effects of the *CYB5R3* 729G allele on gene expression and enzyme activity.

Methodology

Genotyping study

Study population and sample collection—To determine the prevalence of this variant in an overrepresented breed, allele and genotype frequencies were compared between Doberman Pinschers (DOBE) and dogs of other breeds (nonDOBE). Whole blood (EDTA or heparin) or buccal mucosal swabs were collected from dogs at the University of Wisconsin Veterinary Medical Teaching Hospital (UW-VMTH) patient population, from dogs owned by School of Veterinary Medicine students, faculty, and staff, at outreach events at local dog training facilities, and through Doberman Pinscher interest group message boards and listservs. All dogs in the DOBE and non DOBE groups were reported by their owners to be naïve to sulfonamide antibiotics.

Samples were also collected from additional dogs with sulfonamide HS and dogs tolerant to a course of sulfonamide antibiotics (TOL). These dogs were recruited through the UW-VMTH or through referral consultation with one of the authors (LT) between 2014 and 2017. All dogs in these groups had received a therapeutic course of potentiated sulfonamide antibiotics. Dogs were included in the HS group if hepatopathy, blood dyscrasia, polyarthropathy, fever, or skin eruption developed five days or longer after initiation of antibiotic therapy and no other plausible explanation for these clinical signs was present. Dogs were included in the TOL group if they completed at least a 14-day course of potentiated sulfonamide antibiotics without developing adverse reactions. *CYB5R3* 729A>G genotyping results from these dogs were combined with those of previously reported HS and TOL dogs (n=34) (Funk-Keenan et al., 2012) and analyzed as a single study population. The study protocol was approved by the University of Wisconsin's Institutional Animal Care and Use Committee (Protocol # V01478-0-02-13).

Genotype determination—Genomic DNA was extracted from blood or buccal swab with a commercial kit (DNEasy Blood and Tissue Kit; Qiagen, Hilden, Germany) and quantified and checked for quality via spectrometry (Take3; BioTek Instruments Inc., Winooski, VT). The region around the *CYB5R3* 729 position was amplified via PCR in 20 µl reactions: 10 µl DNA polymerase master mix (AmpliTaq Gold or DreamTaq; Thermo Fisher Scientific, Madison, WI), 1 µl genomic DNA template, 0.5 µM forward primer (5' TCCTCAGGTGAGATGGGGAA 3'), 0.5 µM reverse primer (5' CAGAAGGCCAGCTCTCTGTC 3'). Cycling conditions were as follows: initial denaturation at 95°C for 5 minutes, then 30 cycles of 94 °C for 15 seconds, 60°C for 30 seconds, 72°C for 1 minutes, with a final extension of 72°C for 7 minutes. Amplicons were checked for size (564 bp) with gel electrophoresis and sequenced via Sanger sequencing through the University of Wisconsin Biotechnology Center. For each dog, genotype was determined by inspection of electrophoretograms.

Functional studies

The hypothesis that *CYB5R3* 729G leads to a more stable and difficult to translate mRNA transcript, thus decreasing protein expression and total enzyme function, was initially investigated in genotyped canine liver samples. The functional implications of this variant were also assessed in an *in vitro* cell transfection and expression system.

Canine hepatic *CYB5R3* expression and function—Genomic DNA was extracted from banked livers of 46 dogs of varying breeds (no Dobermans included) and genotyped for *CYB5R3* 729A>G. The dogs included 24 from our laboratory (18 purpose bred hounds and 6 beagles), and 22 provided by Dr. Michael Court at Washington State University (9 mixed breed dogs, 5 greyhounds, 4 Chihuahuas, 3 beagles, and 1 Labrador retriever). Livers with homozygous AA and GG genotypes were used to compare *CYB5R3* mRNA expression, microsomal cytochrome *b₅* reductase (b5R) protein expression, and microsomal sulfamethoxazole-hydroxylamine reduction activity. mRNA expression was determined using qRT-PCR: total RNA was extracted from canine livers with a commercial kit (RNEasy Mini Plus; Qiagen, Hilden, Germany) and digested for genomic DNA (TURBO DNA-free; Thermo Fisher Scientific, Madison, WI). RNA was quantified (Take3; BioTek Instruments

Inc., Winooski, VT) and assessed for quality (2100 Bioanalyzer; Agilent Technologies, Santa Clara, CA); all samples had RNA integrity numbers of 6 or greater (Fleige & Pfaffl, 2006). cDNA was generated from 500 ng of RNA via reverse transcription with random oligomers (Superscript IV VILO cDNA Synthesis Kit; Thermo Fisher Scientific; Madison, WI). Primers were designed for the canine *CYB5R3* transcript along with two canine reference genes (*B2M*, *HPRT1*, Supplemental Data) and primer efficiencies determined for each using a 10-fold dilution series of pooled cDNA. SYBR Green (LightCycler 480 SYBR Green I Master; Roche Diagnostics; Indianapolis, IN) qPCR with melting curves was performed on the LightCycler 96 Instrument (Roche; Diagnostics; Indianapolis, IN) with parameters adapted from manufacturer's directions. mRNA expression data were reported as normalized ratios.

For quantification of b5R protein expression and enzyme function, canine liver microsomes were prepared using ultracentrifugation. Protein expression was determined by immunoblotting using calnexin as a loading control. For each sample, 0.625 µg of microsomal protein was electrophoresed in polyacrylamide and transferred onto a nitrocellulose membrane. This loading amount yielded adequate resolution for relative quantification for all target proteins. Membranes were blocked in 5% bovine serum albumin (Sigma-Aldrich; St. Louis, MO) for 1 hour, washed in 0.1% PBS-Tween, and then incubated with polyclonal rabbit anti-dog calnexin antibody (1:2,000; ab75801, Abcam, Cambridge, United Kingdom) and polyclonal rabbit anti-human b5R antibody (1:20,000) (Kurian et al., 2004) for 1 hour at room temperature. The membrane was washed 3 times and incubated with horseradish peroxidase-conjugated rabbit anti-IgG antibody (1:5,000; Promega Corp., Fitchburg, WI) in 1% milk for 1 hour at room temperature and washed again. Protein bands were detected by chemiluminescence (ECL Western Blotting Detection Reagent; GE Healthcare, Buckinghamshire, United Kingdom). Band densitometry was assessed using ImageJ (National Institutes of Health, Bethesda, MD) and protein expression was reported as b5R:calnexin density ratio.

Microsomal b5R enzyme activity was assessed by measuring reduction of sulfamethoxazole-hydroxylamine (SMX-HA; Dalton Chemical Co., Toronto, Ontario, Canada) to sulfamethoxazole (SMX; Sigma-Aldrich; St. Louis, MO) by HPLC as previously described, (Trepanier & Miller, 2000) using 125 µg microsomal protein and 200 µM SMX-HA. Enzyme activities were expressed as nmoles SMX generated/mg microsomal protein/min.

***In vitro* canine *CYB5R3* expression**—The full length *CYB5R3* cDNA sequence was amplified from the liver cDNA of a dog homozygous for the reference 729A allele and cloned into a mammalian expression vector (pcDNA3.1(+); Invitrogen Corp., Carlsbad, CA). A 729G variant-containing plasmid was generated via site-directed mutagenesis (QuickChange Multi Site-Directed Mutagenesis Kit; Agilent Technologies, Santa Clara, CA) with the following primers: forward 5' GTGGACAAAGCCCCGGAAGCCTGGGACTA 3', reverse 5' TAGTCCCAGGCTTCCGGGGCTTTGTCCAC 3'. Both the A- and G-containing plasmids were transfected into HepG2 cells (ATCC, Manassas, VA), using a cationic transfection reagent (FuGENE HD; Promega Corp., Fitchburg, WI). Cells were incubated for 48 hours at 37°C, 5% CO₂ prior to downstream applications.

In order to ensure that transfection efficiency was similar between 729A- and G-plasmid transfected cells, HepG2 cells were co-transfected with a plasmid expressing green fluorescent protein (pmaxGFP; Lonza Group, Basel, Switzerland). In separate experiments, transfection efficiencies were determined by counting GFP-expressing cells normalized to the total number of cells in each culture as determined by SYTOX Orange (Thermo Fisher Scientific; Madison, WI) counterstaining. Cell counts were assessed in ImageJ (National Institutes of Health, Bethesda, MD). There was no significant difference in transfection efficiencies between A- and G-plasmid transfected cells ($10.6 \pm 6.5\%$ vs. $12.5 \pm 6.6\%$, $p = 0.33$ using a Student's t-test).

Following cell transfection and incubation, RNA was extracted from HepG2 cells for cDNA generation and protein was extracted for quantification. mRNA expression was assessed by qPCR using primers for canine *CYB5R3* with human *B2M* and *HMBS* as reference genes (Supplemental Data). Protein was extracted from separate cell culture preparations using passive lysis buffer (Promega Corp., Fitchburg, WI) and quantified via immunoblotting. For each sample, 2.5 μ g of lysate protein was electrophoresed in polyacrylamide and transferred onto a nitrocellulose membrane. Membranes were blocked in 5% bovine serum albumin for 1 hour, washed, and incubated with anti-mouse β -actin monoclonal antibody conjugated with horseradish peroxidase (1:5,000; ab20272, Abcam, Cambridge, United Kingdom) for 1 hour at room temperature. β -actin protein bands were detected by chemiluminescence and then the membrane was stripped (Restore PLUS Western Blot Stripping Buffer; ThermoScientific, Rockford, IL). The membrane was incubated with polyclonal rabbit anti-human b5R antibody (1:50,000 in 1% milk) for 1 hour at room temperature, followed by horseradish peroxidase-conjugated (1:5,000 in 1% milk) for 1 hour at room temperature and detection of b5R protein bands. Band densitometry was assessed using ImageJ and protein expression was reported as b5R: β -actin density ratio.

Statistical analyses

Continuous data were expressed as median [range]. Differences in age and sex between groups were compared with the Mann-Whitney U and Chi-square tests, respectively. Allele and genotype frequencies were compared between groups using Fisher's exact and Chi-square tests, respectively. Expression levels and activities were compared between AA vs. GG genotype livers and A- vs. G-plasmid transfected cells using the Mann-Whitney U test. All analyses were performed using commercial software (Prism; GraphPad Software Inc., San Diego, CA). Statistical significance was set at $p < 0.05$.

Results

Genotyping

Eighty-four dogs were included in the breed comparison of allele frequencies (24 DOBE, 60 nonDOBE). Demographic data are reported in Table 1. There was no significant difference in age ($p = 0.08$) between DOBE and nonDOBE dogs. However, intact animals were overrepresented in the DOBE group compared to the nonDOBE group ($p = 0.0005$). The G allele was significantly over represented in DOBE dogs (1.00) compared to nonDOBE dogs (0.567, $p < 0.0001$, Table 1). Similarly, the GG genotype was overrepresented in DOBE

dogs (1.00) compared to nonDOBE dogs (0.417, $p < 0.0001$). *CYB5R3* 729A>G allele and genotype frequencies were also compared between dogs hypersensitive and tolerant to sulfonamide antibiotics (24 HS, 20 TOL). Of these, 34 were previously reported (Funk-Keenan et al., 2012) and 10 were newly recruited dogs. There was no significant difference in age ($p = 0.39$) or sex ($p = 0.69$) between groups (Table 1). The G allele was significantly over-represented in HS dogs (0.792) compared to TOL dogs (0.550, $p = 0.022$). The GG genotype frequency appeared higher in the HS dogs (0.625) vs. the TOL dogs (0.300), but this difference did not reach statistical significance ($p = 0.06$). Genotyping and demographic data for all individual subjects are provided in Supplemental Data.

Functional effects of *CYB5R3* 729G: canine liver expression and activity

Forty-six banked dog livers of adequate RNA quality were available for analysis. Of these, 12 were 729AA and 15 were 729GG; 19 were heterozygotes that were not further phenotyped. Expression of *CYB5R3* mRNA was not significantly different between AA and GG livers (normalized ratio, 1.16 [0.17 – 1.62] vs. 0.97 [0.19 – 1.72], $p = 0.17$, Figure 1A). Similarly, there was no significant difference between b5R protein expression in microsomes of AA vs. GG livers (0.746 [0.437 – 1.37] vs. 0.593 [0.303 – 1.22], $p = 0.427$, Figure 1B). Because expression of the loading control was somewhat variable between samples, b5R protein expression was also compared without normalization to calnexin, with similar results comparing AA and GG livers (0.826 [0.517 – 1.20] vs. 0.628 [0.367 – 1.13], $p = 0.075$). Finally, microsomal SMX-hydroxylamine reduction activities did not differ between AA and GG livers (1.01 [0.685 – 1.72] vs. 0.973 [0.530 – 1.64] nmol/min/mg, $p = 0.373$, Figure 1C).

Functional effects of *CYB5R3* 729G: in vitro expression

In transfection experiments, there was no significant difference in expression of canine *CYB5R3* mRNA between HepG2 cells transfected with the A vs. G allele (normalized ratio, 4391 [2499 – 7936] vs. 4867 [4127 – 9484], $p = 0.31$, Figure 2A). Similarly, b5R protein expression did not differ in cells transfected with the A vs. G allele (5.06 [4.43 – 7.38] vs. 5.93 [2.88 – 11.0], $p = 1.00$, Figures 2B).

Discussion

Cytochrome *b*₅ reductase, along with cytochrome *b*₅, catalyzes the reduction of the reactive sulfonamide metabolite, SMX hydroxylamine, back to the stable parent drug (Kurian et al., 2004; Trepanier & Miller, 2000). Since the hydroxylamine metabolite contributes to the immunogenicity of sulfonamide antibiotics such as sulfamethoxazole and sulfadiazine (Cribb & Spielberg, 1990; Farrell et al., 2003; Shear & Spielberg, 1985), hydroxylamine reduction should be protective against sulfonamide HS. Dogs may rely more heavily on the cytochrome *b*₅ reductase system for sulfonamide detoxification, because dogs lack the other major detoxifying pathway employed by humans, the *N*-acetyltransferases (Trepanier et al., 1997).

We previously examined the role of variants in the cytochrome *b*₅ reductase (*CYB5R3*) and cytochrome *b*₅ (*CYB5A*) genes in the risk of sulfonamide HS in dogs. We found a synonymous SNP in the *CYB5R3* gene, 729A>G, that was significantly associated with

sulfonamide HS in a group of 18 affected and 16 control dogs (Funk-Keenan et al., 2012). The purpose of this study was to determine whether the risk allele was over-represented in Doberman Pinschers, and to evaluate the effects of the *CYB5R3*G allele on gene expression and enzyme activity.

All 24 Doberman Pinschers (sulfonamide naïve) were homozygous for the 729G allele. If this variant is truly associated with sulfonamide HS, then this may contribute to the apparent breed risk in the Doberman Pinscher. However, not all Dobermans exposed to sulfonamide antibiotics develop a HS reaction; in fact, one of our drug-tolerant controls was a Doberman Pinscher. Therefore, other factors clearly influence the risk of sulfonamide HS. Further evaluation and genotyping of Dobermans tolerant and hypersensitive to sulfonamide antibiotics is warranted to identify other possible genetic risk factors that might contribute to risk.

In a population of dogs expanded from our original report, (Funk-Keenan et al., 2012) the *CYB5R3* 729G allele remained over-represented in HS dogs (0.792) compared to dogs tolerant to sulfonamide antibiotics (0.550); thus, the G allele may indeed predispose to sulfonamide HS. However, it is important to note that 30% of tolerant dogs are still homozygous for 729GG. Therefore, it follows that carriage of the *CYB5R3* 729G allele is not sufficient as a single risk factor for sulfonamide HS.

We next investigated the effects of *CYB5R3* 729G on gene expression and activity in both canine liver and an *in vitro* mammalian expression system. The *CYB5R3* 729A>G variant is a synonymous coding SNP, so it should not affect enzyme structure or function. However, based on *in silico* predictions, the G allele may confer a more stable and difficult to translate structure to the mRNA transcript (Funk-Keenan et al., 2012). Therefore, we hypothesized that 729G might lead to increased mRNA levels but decreased protein synthesis and SMX-HA reduction.

We found no differences in *CYB5R3* mRNA or b5R protein expression between canine livers of 729AA vs. GG genotype. However, the loading control (calnexin) used for immunoblotting was somewhat variable between samples so b5R protein immunoreactivity was analyzed without normalization. These results also showed no significant difference in b5R expression between groups ($p = 0.075$) and, even if these findings were significant, the small difference in expression (~25% decrease in GG livers) is unlikely to have a pharmacologically meaningful effect on drug metabolism. This is corroborated by endogenous SMX-hydroxylamine reduction, which did not differ between genotypes, even at lower substrate concentrations (10 μ M SMX-hydroxylamine, data not shown). In addition, a direct effect of the 729G allele on protein expression was not confirmed by *in vitro* transfection in HepG2 cells. Of note, SMX-hydroxylamine reduction could not be reliably measured in transfected HepG2 cells over background activities observed in non-transfected cells (data not shown), which may reflect a requirement for parallel co-transfection of *CYB5A* at an optimized stoichiometry (Kurian et al., 2004), which was not pursued.

Taken together, we found no evidence that the *CYB5R3* 729G allele decreases SMX-hydroxylamine reduction and detoxification. However, this allele is associated with a HS

clinical phenotype in dogs. This could be a false positive association, or this variant could be in linkage disequilibrium with another gene locus which does cause functional alterations that influence clinical phenotype.

This study was limited by a small sample size for genotyping experiments. As an idiosyncratic reaction, sulfonamide HS is intrinsically an uncommon occurrence (Noli et al., 1995); furthermore, sulfonamides are less commonly used today because other antibiotics with lower risks of adverse effects are available. However, as antimicrobial resistance to “safer” antibiotics (e.g. beta-lactams, fluoroquinolones) increases, we may see increased sulfonamide use and a resurgence of HS reactions. Therefore, it is important to understand this idiosyncratic reaction and its risk factors as best as possible.

In conclusion, all Doberman Pinschers genotyped were homozygous for the *CYB5R3729G* allele, which is over-represented in dogs with sulfonamide HS. However, we could find no evidence that this variant leads to functionally impaired detoxification of sulfamethoxazole hydroxylamine. Further work is needed to explore risk factors for sulfonamide HS in dogs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

b5R	cytochrome <i>b</i> ₅ reductase
HRP	horseradish peroxidase
HS	hypersensitivity
SMX	sulfamethoxazole
SNP	single nucleotide polymorphism

References

- Cribb AE. Idiosyncratic reactions to sulfonamides in dogs. *Journal of the American Veterinary Medical Association*. 1989; 195:1612–1614. [PubMed: 2532190]
- Cribb AE, Spielberg SP. An in vitro investigation of predisposition to sulphonamide idiosyncratic toxicity in dogs. *Veterinary Research Communications*. 1990; 14:241–252. [PubMed: 2382408]
- Farrell J, Naisbitt DJ, Drummond NS, Depta JPH, Vilar FJ, Pirmohamed M, Park BK. Characterization of sulfamethoxazole and sulfamethoxazole metabolite-specific T-cell responses in animals and humans. *Journal of Pharmacology and Experimental Therapeutics*. 2003; 306:229–237. [PubMed: 12676884]

- Fleige S, Pfaffl MW. RNA integrity and the effect on the real-time qRT-PCR performance. *Molecular Aspects of Medicine*. 2006; 27:126–139. [PubMed: 16469371]
- Fox LE, Ford S, Alleman AR, Homer BL, Harvey JW. Aplastic anemia associated with prolonged high-dose trimethoprim-sulfadiazine administration in two dogs. *Veterinary Clinical Pathology*. 1993; 22:89–92. [PubMed: 12669295]
- Funk-Keenan J, Sacco J, Wong YYA, Rasmussen S, Motsinger-Reif A, Trepanier LA. Evaluation of polymorphisms in the sulfonamide detoxification genes CYB5A and CYB5R3 in dogs with sulfonamide hypersensitivity. *Journal of Veterinary Internal Medicine*. 2012; 26(5):1126–1133. [PubMed: 22816446]
- Giger U, Werner LL, Millichamp NJ, Gorman NT. Sulfadiazine-induced allergy in six Doberman pinschers. *Journal of the American Veterinary Medical Association*. 1985; 186:479–484. [PubMed: 3972708]
- Gray A. Trimethoprim-sulphonamide hypersensitivity in dogs. *Veterinary Record*. 1990; 127:579–580.
- Harvey JW. Pathogenesis, laboratory diagnosis, and clinical implications of erythrocyte enzyme deficiencies in dogs, cats, and horses. *Veterinary Clinical Pathology*. 2006; 35:144–156. [PubMed: 16783707]
- Kurian JR, Bajad SU, Miller JL, Chin NA, Trepanier LA. NADH cytochrome b5 reductase and cytochrome b5 catalyze the microsomal reduction of xenobiotic hydroxylamines and amidoximes in humans. *Journal of Pharmacology and Experimental Therapeutics*. 2004; 311:1171–1178. [PubMed: 15302896]
- Mohanasundaram J, Rajaram S, Mohanasundaram S. Familial tendency in hypersensitivity reactions to co-trimoxazole. *Journal of the Indian Medical Association*. 1998; 96:21–22. [PubMed: 9601187]
- Neuman MG, Malkiewicz IM, Shear NH. A novel lymphocyte toxicity assay to assess drug hypersensitivity syndromes. *Clinical biochemistry*. 2000; 33:517–524. [PubMed: 11124336]
- Noli C, Koeman JP, Willemse T. A retrospective evaluation of adverse reactions to trimethoprim-sulphonamide combinations in dogs and cats. *Veterinary Quarterly*. 1995; 17:123–128. [PubMed: 8751272]
- Shear NH, Spielberg SP. In vitro evaluation of a toxic metabolite of sulfadiazine. *Can J Physiol Pharmacol*. 1985; 63:1370–1372. [PubMed: 4075255]
- Shear NH, Spielberg SP, Grant DM, Tang BK, Kalow W. Differences in metabolism of sulfonamides predisposing to idiosyncratic toxicity. *Annals of Internal Medicine*. 1986; 105:179–184. [PubMed: 3729200]
- Trepanier LA. Idiosyncratic toxicity associated with potentiated sulfonamides in the dog. *Journal of veterinary pharmacology and therapeutics*. 2004; 27:129–138. [PubMed: 15189298]
- Trepanier LA, Miller JL. NADH-dependent reduction of sulphamethoxazole hydroxylamine in dog and human liver microsomes. *Xenobiotica*. 2000; 30:1111–1121. [PubMed: 11307968]
- Trepanier LA, Danhof R, Toll J, Watrous D. Clinical findings in 40 dogs with hypersensitivity associated with administration of potentiated sulfonamides. *Journal of Veterinary Internal Medicine*. 2003; 17:647–652. [PubMed: 14529130]
- Trepanier LA, Ray K, Winand NJ, Spielberg SP, Cribb AE. Cytosolic arylamine N-acetyltransferase (NAT) deficiency in the dog and other canids due to an absence of NAT genes. *Biochemical pharmacology*. 1997; 54:73–80. [PubMed: 9296352]
- Werner LL, Bright JM. Drug-induced immune hypersensitivity disorders in two dogs treated with trimethoprim sulfadiazine: case reports and drug challenge studies. *Journal of the American Animal Hospital Association*. 1983; 19:783–790.
- Whur P. Possible trimethoprim-sulphonamide induced polyarthritis. *The Veterinary record*. 1987; 121:91–92.

Figure 1.

CYB5R3 mRNA expression (A), b5R protein expression (B), and sulfamethoxazole (SMX) hydroxylamine reduction (C) in canine livers homozygous for *CYB5R3* 729A (n = 12) and *CYB5R3* 729G (n = 15). *Panel B insert:* immunoblotting of canine b5R expression in canine microsomes of dogs homozygous for the A and G allele at the *CYB5R3* 729A>G SNP. Calnexin was used as a loading control.

Figure 2.

CYB5R3 mRNA (A) and cytochrome *b*₅ reductase (b5R) protein expression (B) in HepG2 cells transfected with the *CYB5R3* gene containing either the 729A or G allele. Five separate preparations of A- and G-plasmid transfected cells were used for mRNA quantification and three preparations were used for protein quantification. There were no significant differences between groups for mRNA or protein expression. *Panel B insert:* immunoblotting of canine b5R expression in HepG2 cells transfected with the *CYB5R3* gene containing either the 729A or G allele. The upper b5R band co-localized with canine b5R and thus was used for densitometry readings. The lower b5R band co-localized with human b5R representing endogenous expression by the HepG2 cells (data not shown). β-actin was used as a loading control.

Table 1Demographic data of dogs included in *CYB5R3* genotyping.

	DOBE n = 24	nonDOBE n = 60	HS n = 24	TOL n = 20
Age (years)	7.3 [0.3 – 12]	5.0 [0.8 – 14]	5.5 [0.5 – 14]	3.0 [0.3 – 12]
Sex	MC 9 FS 6 MI 5 FI 4	MC 33 FS 25 MI 1 FI 1	MC 5 FS 16 MI 2 FI 1	MC 6 FS 10 MI 2 FI 2
Breed (# of dogs)	Doberman Pinscher (24)	Mixed breed (25), Golden retriever (5), German Shepherd (5), Australian Shepherd (4), Labrador retriever (4), Australian Cattle Dog (3), Chihuahua (2), Cavalier King Charles Spaniel (2), Border collie (2); 1 each: Boxer, English Bulldog, Brittany Spaniel, Cocker Spaniel, Plott Hound, Chow Chow, Bernese Mountain Dog, Mallinois	Labrador retriever (6), Golden retriever (5), Great Dane (3), Mixed breed (3), Samoyed (2); 1 each: German Shepherd, Doberman Pinscher, Malamute, Springer Spaniel, Dalmatian	Mixed breed (6), Boxer (2), Cocker Spaniel (2); 1 each: Australian Shepherd, Poodle, Shar Pei, Labrador retriever, German Shepherd, Brittany Spaniel, Shih Tzu, Doberman Pinscher, Bernese Mountain Dog, Golden retriever
<i>CYB5R3</i> 729A>G Allele Frequency				
A	0.00	0.433	0.208	0.450
G	1.00[*]	0.567	0.792^{**}	0.550
<i>CYB5R3</i> 729A>G Genotype Frequency				
AA	0.00	0.283	0.042	0.200
AG	0.00	0.300	0.333	0.500
GG	1.00	0.417	0.625	0.300

MC = male castrated, FS = female spayed, MI = male intact, FI = female intact.

^{*} = significantly different from nonDOBE, $p < 0.0001$;^{**} = significantly different from TOL, $p = 0.022$.