Frequency of the nt230 (del4) *MDR1* mutation in Collies and related dog breeds in Germany

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Geyer, J., Döring, B., Godoy, J. R., Leidolf, R., Moritz, A., Petzinger, E. Frequency of the nt230 (del4) *MDR1* mutation in Collies and related dog breeds in Germany. *J. vet. Pharmacol. Therap.* **28**, 545–551.

MDR1 (*ABCB1*) P-glycoprotein exerts a protective function in the blood–brain barrier thereby limiting the entry of many drugs and other xenobiotics to the central nervous system. A nonsense mutation has been described for Collies and related dog breeds which abolishes this function and is associated with increased susceptibility to neurotoxic side effects of several drugs including ivermectin, moxidectin and loperamide. In order to evaluate the occurrence and frequency of this nt230 (del4) *MDR1* mutation in Germany, we screened 1500 dogs. Frequency of the homozygous mutated genotype was highest for Collies (33.0%), followed by Australian Shepherd (6.9%) and Shetland Sheepdog (5.7%). Thirty-seven percent of the Wäller dogs and 12.5% of the Old English Sheepdogs were heterozygous for the mutant *MDR1* (−) allele. Considering the predominant role of *MDR1* P-glycoprotein in drug disposition and in particular for blood–brain barrier protection, *MDR1* genotype-based breeding programs are recommended for improving the safety of drug therapy in these canine breeds.

(Paper received 10 May 2005; accepted for publication 25 August 2005)

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INTRODUCTION

The multidrug resistance (MDR) transporter P-glycoprotein (P-gp), the product of the *MDR1* (*ABCB1*) gene, belongs to the family of membrane-bound ATP-binding cassette (ABC) transporters (Dean *et al.*, 2001). P-gp acts as an ATP-dependent efflux pump which transports a wide range of structurally unrelated hydrophobic and amphipathic drugs, toxins, and xenobiotics (Borst & Oude-Elferink, 2002). Juliano and Ling (1976) isolated P-gp from chemotherapeutic drug-resistant Chinese hamster ovary cells and identified this protein as a major part of the functional MDR of these cells. In addition, P-gp is expressed in healthy tissues with secretory/excretory function such as the liver (canalicular membrane of hepatocytes), kidney (luminal membrane of proximal tubules), and the intestine (brush border membrane of enterocytes), as well as in capillaries of the brain and testis, trophoblasts in the placenta, and hematopoietic stem cells (Thiebaut *et al.*, 1987; Cordon-Cardo *et al.*, 1989, 1990; Chaudhary & Roninson, 1991). Apical/luminal expression of P-gp in these tissues limits drug absorption from the intestine, promotes drug elimination into bile, urine, and intestine, and restricts drug entry into the central nervous system (CNS). It is now well accepted that an essential function of P-gp in non-neoplastic tissues is to confer an intrinsic MDR by eliminating potentially toxic xenobiotics from the body and preventing their entry into the brain and organs of reproduction (Schinkel, 1997; Fromm, 2000; Tanigawara, 2000).

The functional importance of P-gp in the blood–brain barrier has been extensively studied in genetically engineered *mdr1a* (−/−) knockout mice, *mdr1a* P-gp-deficient CF-1 mice, and porcine brain capillary cells (Schinkel *et al.*, 1994; Kwei *et al.*, 1999; Nobmann *et al.*, 2001). *Mdr1a* (−/−) knockout mice are healthy and fertile under laboratory conditions, but they have increased sensitivity to drugs known to be P-gp substrates. An example is ivermectin, a widely used anthelmintic and acaricide drug, which is an excellent P-gp substrate (Schinkel *et al.*, 1994; Lankas *et al.*, 1997; Kwei *et al.*, 1999). Ivermectin acts as an agonist of a family of invertebrate-specific glutamate-gated chloride ion channels which are phylogenetically related to vertebrate $GABA_A$-gated chloride channels (Edwards, 2003). Normally, plasma ivermectin concentrations exceed brain concentrations by about 10-fold in mammals, because P-gp limits the passage of ivermectin across the blood–brain barrier (Schinkel *et al.*, 1994). Therefore, the contact of ivermectin with mammalian GABA-sensitive neurons which are broadly expressed in the CNS is highly restricted. However, disruption of the *mdr1a* gene in *mdr1a* (−/−) knockout mice leads to 87-fold higher concentrations of ivermectin in the brain associated with

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50–150 times increased toxicity ($LD_{50} = 0.7–0.8$ mg/kg vs. 50–60 mg/kg) over that in wild-type mice (Schinkel *et al.*, 1994).

Similar to the *mdr1a* (−/−) mice, it is well known in veterinary medicine that a subpopulation of Collie dogs shows increased neurotoxicity to ivermectin and it was speculated that this effect may be caused by increased brain penetration in these dogs (Pulliam *et al.*, 1985). In the literature, the name 'ivermectin-sensitive Collie' has been introduced to describe these dogs, which show when exposed to 120–150 μg/kg ivermectin orally clear signs of ivermectin toxicosis, including apparent depression, ataxia, somnolence, mydriasis, salivation, and tremor (Paul *et al.*, 1987; Tranquilli *et al.*, 1989; Vaughn *et al.*, 1989; Hopper *et al.*, 2002). In contrast, ivermectin can be administered at a dosage of 2.5 mg/kg with no evidence of toxicosis in Beagles and ivermectin nonsensitive Collies (Pulliam *et al.*, 1985). Within the last 4 years, two groups have independently identified a 4-bp deletion in the *MDR1* gene of an ivermectin-sensitive Collie (Mealey *et al.*, 2001; Roulet *et al.*, 2003). This nt230 (del4) *MDR1* deletion involves a frame shift at amino acid position 75 followed by a premature stop codon at amino acid position 91. This severely truncated protein is nonfunctional and was not detectable by Western blot analysis in ivermectin-sensitive Collies (Roulet *et al.*, 2003). Furthermore, Mealey *et al.* (2001) demonstrated in 17 Collies that all dogs with a homozygous *MDR1* mutation showed the ivermectin-sensitive phenotype but none of the Collies with a heterozygous *MDR1* mutation or the wild-type genotype, pointing to an autosomal recessive inheritance pattern.

Very recently, Neff *et al.* (2004) performed a genetic screening in dog breeds from the US and identified not only Collies, but also Australian Shepherd, English Shepherd, Longhaired Whippet, McNab, Old English Sheepdog, Shetland Sheepdog, and Silken Windhound to be affected from this *MDR1* mutation. However, nothing is known about the breed distribution and frequency of this particular *MDR1* mutation in dogs from Germany. Therefore, we screened 1500 animals of the Collie, Australian Shepherd, Shetland Sheepdog, Bearded Collie, Border Collie, Old English Sheepdog, and Wäller breeds for the *MDR1* nt230 (del4) mutation using a polymerase chain reaction (PCR)-based diagnostic test (Geyer *et al.*, 2005).

MATERIALS AND METHODS

Animals

Blood samples were received from 1500 client-owned dogs and analyzed for the nt230 (del4) *MDR1* mutation as part of the diagnostic research service of our institute. Data of 578 rough- and smooth-coated Collies, 29 Bearded Collies, 334 Border Collies, 140 Shetland Sheepdogs, 333 Australian Shepherds, 24 Old English Sheepdogs, and 62 Wäller are documented in this study.

PCR-based detection of the nt230 (del4) MDR1 mutation

Genomic DNA was isolated from 200 μL of ethylenediaminetetraacetic acid (EDTA)-anticoagulated blood and PCR amplification was performed in the presence of 100 ng of template DNA as described previously (Geyer *et al.*, 2005). The PCR generated a 138-bp fragment from the wild-type *MDR1* allele and a 134-bp fragment from the mutated *MDR1* allele. Discrimination of these two fragments was performed by high-resolution polyacrylamide gel electrophoresis (6.5% in 1X Tris–borate–EDTA buffer).

RESULTS

A screening of the canine genome, which recently became available in the GenBank/EBI/DDBJ databases, revealed that the canine *MDR1* gene is located on chromosome 14 and is composed of 28 exons. As shown in Fig. 1, the nt230 (del4) *MDR1* mutation was localized to exon 4.

Genotyping for the *MDR1* nt230 (del4) mutation was carried out with a PCR-based method discriminating the mutant allele

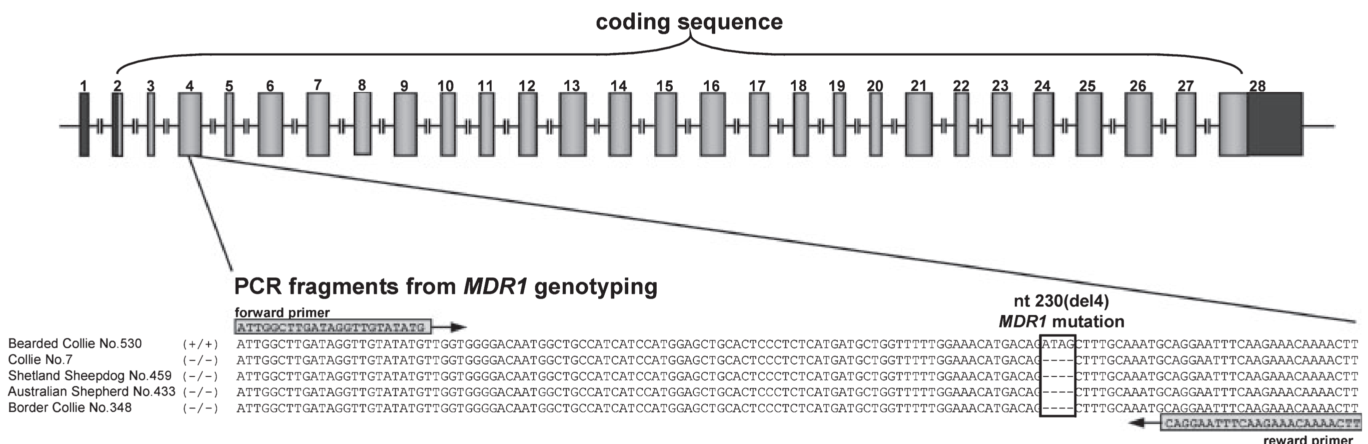

Fig. 1. Exon–intron organization of the canine *MDR1* gene according to GenBank Accession nos. NM_001003215 (mRNA) and NC_006596 (*Canis familiaris* chromosome 14, whole genome shotgun sequence). The nt230 (del4) *MDR1* mutation was localized to exon 4. Primer sequences for *MDR1* genotyping are indicated. PCR fragments of Bearded Collie no. 530, Collie no. 7, Shetland Sheepdog no. 459, Australian Shepherd no. 433, and Border Collie no. 348 were verified by DNA sequencing.

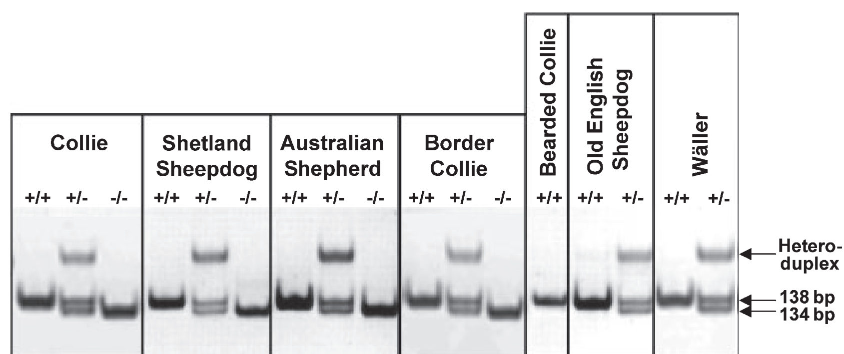

Fig. 2. Representative data from *MDR1* genotyping of 1500 dogs from Germany. DNA was isolated from whole blood, and subjected to polymerase chain reaction (PCR) and polyacrylamide gel electrophoresis. Lengths of the PCR fragments from the wild-type *MDR1* allele (+) and the mutated *MDR1* alleles (−) were 138- and 134-bp, respectively. In the case of a heterozygous *MDR1* mutation (+/−) fragments of both sizes are visible and an additional heteroduplex with markedly decreased mobility in the gel. The nt230 (del4) *MDR1* mutation was detected for Collie, Shetland Sheepdog, Australian Shepherd, Border Collie, Old English Sheepdog and Wäller, but not for Bearded Collie.

Table 1. Frequencies of the nt230 (del4) *MDR1* mutation in 1500 dogs in Germany

		Genotype (%)			
Race	Allele (%)	MDR1 (+/+)	MDR1 (+/−)	MDR1 (−/−)	*n*
Collie	54.6	23.9	43.1	33.0	578
Shetland Sheepdog	30.0	45.7	48.6	5.7	140
Australian Shepherd	19.5	67.9	25.2	6.9	333
Wäller	18.5	62.9	37.1	0	62
Old English Sheepdog	6.3	87.5	12.5	0	24
Border Collie	0.6	99.1	0.6	0.3	334
Bearded Collie	0	100	0	0	29

(134-bp) from the wild-type allele (138-bp). This method is feasible for Collie, Australian Shepherd, Shetland Sheepdog, Border Collie, Bearded Collie, Old English Sheepdog, and Wäller (Fig. 2).

The frequency of the mutated *MDR1* allele varied markedly between these breeds and was highest for Collies (54.6%), followed by Shetland Sheepdog (30.0%), Australian Shepherd (19.5%), Wäller (18.5%), Old English Sheepdog (6.3%), and Border Collie (0.6%) (Table 1). Accordingly, 23.9% of the Collies, 45.7% of the Shetland Sheepdogs, 67.9% of the Australian Shepherds, 62.9% of the Wäller, and 87.5% of the Old English Sheepdogs were tested for homozygous intact *MDR1* alleles. In 334 Border Collies only one dog with homozygous and two dogs with heterozygous mutated *MDR1* alleles were identified, indicating a very rare occurrence of the nt230 (del4) *MDR1* mutation in this breed. Only few samples were obtained from Bearded Collies. All tested dogs of this breed exhibited the intact *MDR1* (+/+) genotype.

DISCUSSION

In vivo relevance of MDR1 in pharmacokinetics

Because of the predominant role of *MDR1* P-gp in drug disposition, mutations of the *MDR1* gene affecting expression and/or function of P-gp will alter the pharmacokinetic properties of many *MDR1* drugs, leading to enhanced oral bioavailability and reduced drug elimination through the liver, kidney, and gut (Sakaeda *et al.*, 2002; Fromm, 2004; Gerloff, 2004; Marzolini *et al.*, 2004). Moreover, the protective function of P-gp is important in the blood–testis barrier and placenta limiting the entry of toxic substances into the gonads and the fetal circulation, respectively (Cordon-Cardo *et al.*, 1989, 1990). However, as demonstrated in ivermectin sensitivity of *mdr1a* (−/−) knockout mice and dogs with *MDR1* (−/−) mutation, the enhanced brain penetration of P-gp substrates is apparently the most relevant clinical consequence of an abolished P-gp function provoking neurotoxic side effects (Pulliam *et al.*, 1985; Schinkel *et al.*, 1994). In addition to ivermectin, a large number of other drugs are well-known substrates of the *MDR1* efflux pump. Some of these were tested in *mdr1a* (−/−) knockout mice and also showed significantly increased brain penetration over normal control mice. These drugs include cyclosporin A, dexamethasone, digoxin, docetaxel, doxorubicin, flesinoxan, grepafloxacin, loperamide, ondansetron, paclitaxel, quinidine, sparfloxacin, tacrolimus, verapamil, and vinblastine (Schinkel *et al.*, 1994, 1995, 1996; Kusuhara *et al.*, 1997; Hendrikse *et al.*, 1998; Yokogawa *et al.*, 1999; Tamai *et al.*, 2000; Zhang *et al.*, 2000; Van der Sandt *et al.*, 2001; Uhr *et al.*, 2002; Kemper *et al.*, 2003, 2004) (Table 2). Therefore, it is very likely that dogs with the *MDR1* (−/−) mutation would also experience greater toxicity with these drugs.

Because of the expected alterations in drug absorption and pharmacokinetic properties, and the enhanced blood–tissue barrier penetration of *MDR1* drugs in these dogs, clinical studies are necessary for a *MDR1* genotype-based dose adjustment to prevent adverse drug reactions particularly for the brain and the gonads. Clinical observations have already indicated that the anti-diarrheal agent loperamide, which does not produce opioidic depressant CNS effects at usual doses, caused severe neurologic toxicosis in Collies with *MDR1* (−/−) genotype at doses safely used in other breeds and human (0.14 mg/kg body weight) (Sartor *et al.*, 2004). Furthermore, increased sensitivity

Table 2. Clinically relevant P-glycoprotein substrates which show enhanced brain concentrations in *mdr1a* (−/−) mice compared with wild-type mice after intravenous injection

Drug	Time after administration	Brain concentration ratio [*mdr1a* (−/−)/*mdr1a* (+/+)]	Reference
Ivermectin*	**24 h**	**87§**	**Schinkel *et al.* (1994)**
Digoxin	**4 h**	**35**	**Schinkel *et al.* (1995)**
Tacrolimus	5 h	34	Yokogawa *et al.* (1999)
Quinidine	10 min	28†	Kusuhara *et al.* (1997)
Flesinoxan	3 h	27	Van der Sandt *et al.* (2001)
Vinblastine	**4 h**	**22**	**Schinkel *et al.* (1994)**
Cyclosporine A	**4 h**	**17**	**Schinkel *et al.* (1995)**
Loperamide*	**4 h**	**13§**	**Schinkel *et al.* (1996)**
Paclitaxel	**24 h**	**12‡**	**Kemper *et al.* (2003)**
Verapamil	**1 h**	**9.5**	**Hendrikse *et al.* (1998)**
Docetaxel	24 h	6.2‡	Kemper *et al.* (2004)
Doxorubicin	**24 h**	**5.0**	**Zhang *et al.* (2000)**
Cortisol	2 h	4.6†,¶	Uhr *et al.* (2002)
Ondansetron	30 min	4.0	Schinkel *et al.* (1996)
Sparfloxacin	2 h	3.8‡	Tamai *et al.* (2000)
Grepafloxacin	2 h	2.6‡	Tamai *et al.* (2000)
Dexamethasone	**4 h**	**2.5**	**Schinkel *et al.* (1995)**

Drugs with common veterinary usage are in bold face.
*Drugs with documented neurotoxicity in dogs with homozygous nt230 (del4) *MDR1* mutation.
†Brain-to-plasma partition coefficient ($K_{p,\ brain,\ ko}/K_{p,\ brain,\ wt}$).
‡*mdr1a,b* (−/−) double knockout mice were used.
§Oral administration.
¶s.c. injection.

to an oral dose of 400 μg/kg moxidectin was reported for an Australian Shepherd with homozygous nt230 (del4) *MDR1* mutation (Geyer *et al.*, 2005). Moxidectin is a synthetically derived macrocyclic lactone which is structurally related to ivermectin and is normally tolerated at a dose of 1000 μg/kg body weight orally, as demonstrated in beagle dogs (Vanapalli *et al.*, 2002). Therefore, drugs with intrinsic neurotoxicity, such as ivermectin, moxidectin or loperamide, should be completely excluded from the treatment of dogs with the *MDR1* (−/−) genotype. However, one exception of this rule would be the treatment with low doses of ivermectin (6 μg/kg) and moxidectin (3 μg/kg) for heartworm prevention, the safety of which has been demonstrated in ivermectin-sensitive Collies (Fassler *et al.*, 1991; Paul *et al.*, 2000).

In human, genetic variations of the human *MDR1* gene have been extensively studied in the last few years. Hitherto, more than 20 single nucleotide polymorphisms (SNPs) have been reported with diverse frequency in different ethnic groups (Ieiri *et al.*, 2004). Only few of these have been shown to affect drug absorption, distribution and elimination, resulting in inter-individual variability in drug response and adverse reactions (Sakaeda *et al.*, 2002; Gerloff, 2004; Marzolini *et al.*, 2004). In spite of the abundance of data for human *MDR1* genotyping, a human null allele has not yet been identified. In particular, the nt230 (del4) corresponding exon 4 region of the human *MDR1* gene differs from the palindromic sequence in the canine gene ($GATAG_{dog}$ and $GATAT_{man}$). In addition, human exon 4 has not been reported to cover SNP-based polymorphisms. Nevertheless, serious adverse events were reported in human following treatment with ivermectin for onchocerciasis control. Fifty-five percent of these cases reported from Cameroon were encephalopathies and could not be explained by *Loa loa* infection alone (Twum-Danso, 2003). One possible explanation of this increased susceptibility to ivermectin, which was concentrated in specific geographic areas, are blood–brain barrier abnormalities caused by *MDR1* mutations (Mackenzie *et al.*, 2003). Therefore, it would not be surprising if a geographically restricted *MDR1* mutation which is comparable with the ivermectin-sensitive phenotype in dogs exists also in man.

Worldwide appearance of the nt230 (del4) MDR1 mutation in Collie breeds

When we commenced *MDR1* genotyping of dogs in Germany nothing was known about the occurrence of the nt230 (del4) mutation in European dogs. From seven dog breeds tested in the present study, Collie (33.0%), Australian Shepherd (6.9%), and Shetland Sheepdog (5.7%) are significantly affected by the homozygous nt230 (del4) *MDR1* mutation. The *MDR1* (−/−) genotype was not detected in Wäller or Old English Sheepdogs, despite a high occurrence of the *MDR1* (−) allele in these breeds. Our results are very similar to the results of other *MDR1* genotyping studies from other countries, which determined the *MDR1* (−) allele frequency in the most affected Collie breed to be 51% in the USA (Neff *et al.*, 2004), 60% in the UK (Neff *et al.*, 2004), 56% in the north-west USA (Mealey *et al.*, 2002), 64% in France (Hugnet *et al.*, 2004), and 56% in Australia (Mealey *et al.*, 2005) (Table 3).

Based on these data, we have to assume a worldwide occurrence of the nt230 (del4) *MDR1* mutation with almost identical breed distribution. As it is very unlikely that the same *MDR1* deletion occurred independently in all affected dog breeds,

Table 3. Frequencies of the nt230 (del4) *MDR1* mutation in geographic Collie subpopulations

	Allele (%)	Genotype (%)				
	MDR1 (−)	MDR1 (+/+)	MDR1 (+/−)	MDR1 (−/−)	*n*	Reference
Germany	54.6	23.9	43.1	33.0	578	This study
USA	51	26	46	28	161	Neff *et al.* (2004)
UK	60	15	51	34	94	Neff *et al.* (2004)
North-west USA	56	23	42	35	40	Mealey *et al.* (2002)
Australia	56	12	64	24	33	Mealey *et al.* (2005)
France	64	20	32	48	25	Hugnet *et al.* (2004)

one has to assume that the nt230 (del4) *MDR1* mutation is phylogenetically very old and had occurred before the related continental breed-lines became separated. This was confirmed by Neff *et al.* (2004), who analyzed the extent of lineage disequilibrium and breed history of Collie dogs. These authors concluded that all dogs carrying the nt230 (del4) *MDR1* mutation are descendent of a dog that lived in the UK in the 1800s before the genetic isolation of breeds started by registry. Today, as a consequence of apparently similar breeding strategies and breed registry, the *MDR1* mutation is detectable in dogs all over the world.

The Wäller breed which was established 1994 in Germany is also affected by the nt230 (del4) *MDR1* mutation. This breed is currently maintained by repeated crossbreeding with Australian Shepherd and briard. As the frequency for the mutated *MDR1* (−) allele is rather high in Australian Shepherds, it is very likely that the *MDR1* mutation was introduced into the Wäller breed by this breed. As the Wäller population is still small, outcrossing of the *MDR1* mutation from this race is feasible and could be achieved within the next few years.

Two of the above-mentioned *MDR1* genotyping studies used buccal swabs as source of genomic DNA preparations. However, only 25 of 83 samples (Hugnet *et al.*, 2004) and 61 of 64 samples (Mealey *et al.*, 2005) could be analyzed because of problems in the shipment of the cheek swab samples and non-adequate quantities of DNA. Moreover, it was noted by Roulet *et al.* (2003) that DNA preparations from mouth cells did not give fully reliable amplification results for *MDR1* genotyping, possibly because of contamination with bacterial DNA and the cross-amplification of bacterial ABC-transporter sequences. Therefore, we decided to use blood samples for DNA preparation and received fully reliable PCR amplification of all 1500 DNA preparations used in this study.

Is enhanced drug susceptibility the only consequence of a MDR1 mutation?

As *mdr1a,b* (−/−) knockout mice are obviously healthy and fertile under laboratory conditions, *mdr1* apparently does not convey life-threatening properties (Schinkel *et al.*, 1994). However, an important physiological function of P-gp is to prevent intracellular accumulation of potentially toxic compounds (Schinkel, 1997). Therefore, it is legitimate to hypothesize that *MDR1*-deficient genotypes in addition to alterations in drug pharmacokinetics are also susceptible to certain diseases. In man *MDR1* SNPs and haplotypes affecting *MDR1* expression have been associated with treatment outcome and/or host susceptibility to renal epithelial tumors (Siegsmund *et al.*, 2002), Balkan endemic nephropathy (Atanasova *et al.*, 2004), Parkinson's disease (Drozdzik *et al.*, 2003), breast cancer (Kafka *et al.*, 2003), inflammatory bowel disease (IBD) (Brant *et al.*, 2003), and ulcerative colitis (Schwab *et al.*, 2003). Furthermore, increased susceptibility for IBD was described for *mdr1a* (−/−) knockout mice. These mice spontaneously developed intestinal inflammation under specific pathogen-free conditions which could be prevented when treated with oral antibiotics (Panwala *et al.*, 1998). Furthermore, it was reported that infection of *mdr1a* (−/−) mice with *Helicobacter bilis* induced diarrhea, weight loss, and IBD before the expected onset of spontaneous IBD (Maggio-Price *et al.*, 2002). These observations have raised speculation that the susceptibility to IBD is due to an intestinal epithelial barrier defect caused by *mdr1* deficiency. Accordingly, a selective advantage of the *mdr1* (+/+) genotype in evolution might have been to possess a defense mechanism against several bacterial toxins. Future clinical studies will be required to clarify whether dogs with homozygous *MDR1* mutation are more susceptible to IBD than other dogs. In conclusion, the Collie breed, in addition to the *mdr1* (−/−) knockout mouse, could be another animal model to test for *MDR1*-related drug discovery and pathologies.

ACKNOWLEDGMENTS

The authors thank Dr B. Boschek and Philip Bridger for critical reading of the manuscript and all of the dog owners who contributed blood samples. Technical help was provided by J. Habicht.

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