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Analysis of the canine *MDR1* gene in the dog breed Elo

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To my family

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Abbreviations

A	adenine
ABC	ATP-binding cassette
ABCB	ATP-binding cassette sub-family B
ACTH	adrenocorticotropin hormone
Ala	alanine
APS	ammonium persulphate
ATP	adenosine 5'-triphosphate
BLAST	basic local alignment search tool
bp	base pairs
C	cytosine
cDNA	complementary deoxyribonucleic acid
CFA	Canis familiaris autosome
CHO	Chow-Chow
cM	centiMorgan
CNS	central nervous system
DAL	Dalmatian
DMSO	dimethyl sulfoxid
DNA	deoxyribonucleic acid
dNTPs	deoxy nucleoside 5’triphosphates (N is A, C, G or T)
EDTA	ethylenediamine tetraacetic acid
EMBL	European Molecular Biology Laboratory
EUR	Eurasian Dog
F	forward
G	guanine
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GLM	general linear model
GSP	German Spitz
het	heterozygous
hom	homozygous

HPA axis	hypothalamic-pituitary-adrenal axis
HSA	Homo sapiens autosome
IRD	infrared dye
JSP	Japanese Spitz
M	molar
Mb	mega base
MDR	multidrug resistance
MGB	minor groove binding
mRNA	messenger ribonucleic acid
NCBI	National Center for Biotechnology information
no.	number
OES	Old English Sheepdog
PCR	polymerase chain reaction
PEK	Pekingese
POM	Pomeranian Dog
PSIC	Position-Specific Independent Counts
qRT-PCR	quantitative Reverse Transcriptase-PCR
R	reverse
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
S.D.	standard deviation
SAS	statistical analysis system
Ser	serine
SNP	single nucleotide polymorphism
T	thymine
T_a	annealing temperature
TBE	tris-borate-ethylenediamine tetraacetic acid
TEMED	N,N,N’,N’-tetramethylenediamine
Thr	threonine

Chapter 1

Introduction

Introduction

The *MDR1* (*multidrug resistance*) gene encodes the transmembrane protein pump P-glycoprotein which is found in several mammalian tissues including the brain, intestine, liver, kidney, placenta and testis. The function of this ATP-dependent drug transporter is limiting the uptake of compounds from the gastrointestinal tract and contributing to their excretion via the liver, kidneys, and intestine. Moreover, P-glycoprotein in the blood-brain-barrier and other blood-tissue barriers protects sensitive organs from exposure to toxic compounds that may have entered the bloodstream. A deletion mutation in the canine *MDR1* gene, mdr1-1Δ mutation, was found to be the cause of multiple drug sensitivity in several dog breeds from the Collie lineage such as the Collie, Australian Shepherd or Old English Sheepdog. This problem is commonly known as 'ivermectin-sensitivity' in the Collie. Affected dogs show severe neurotoxic adverse effects when exposed to drugs which are substrates of P-glycoprotein because of an increased permeability of the blood-brain-barrier caused by a nonfunctional P-glycoprotein. Additionally, elevated levels of cortisol can pass through the damaged blood-brain-barrier provoking suppression of the hypothalamic-pituitary-adrenal-axis, thereby resulting in reduced production of hormones. The prevalence of the mdr1-1Δ mutation in affected dog breeds ranges between 0.6 and 64% depending on breed and its geographic subpopulation. Previous studies showed that the mdr1-1Δ allele is identical by descent among affected breeds as evidenced by a single ancestral haplotype. An autosomal recessive inheritance pattern was revealed. No further sequence variations in the canine *MDR1* gene have been reported in the literature as yet, whereas in the orthologous human *ABCB1* (*MDR1*) gene, numerous single nucleotide polymorphisms were described partly associated with decreased amount or functionality of P-glycoprotein.

The Elo is a newly developed German companion dog breed. Breeding of the Elo started in 1987 on the basis of 16 founder animals stemming from nine different dog breeds as following: Eurasian Dog, Old English Sheepdog, Chow-Chow, Samoyed, Dalmatian, Pekingese, Pomeranian Dog, German Spitz and Japanese Spitz. It is not known whether the four Old English Sheepdog founders carried the mdr1-1Δ allele.

The objective of the present study was to analyse the canine *MDR1* gene in the dog breed Elo for the mdr1-1Δ mutation originating in the Old English Sheepdog breed as well as to search

for functional polymorphisms in the gene. In order to achieve this goal, genomic DNA of a representative sample of Elo dogs was screened for the mdr1-1Δ mutation using a specific PCR-based test. Furthermore, a haplotype analysis of the *MDR1* flanking region of the sampled Elo dogs was made to determine whether haplotypes of the Old English Sheepdog breed, which had been found to be associated with the mutant mdr1-1Δ allele, could be recovered in the Elo breed. Finally, the search for functional polymorphisms which might have impact on expression or function of *MDR1* was performed in three exons of the canine *MDR1* gene.

Overview of chapter contents

Chapter 2 reviews the literature for the mdr1-1Δ mutation in dogs including prevalence, genetic aspects and adverse effects of the mdr1-1Δ mutation in affected dog breeds. Clinical signs and P-glycoprotein substrates used in drug therapy of dogs are given. Relevant aspects for the design of breeding strategies are discussed.

Chapter 3 contains the analysis of a representative sample of Elo dogs for the mdr1-1Δ mutation and the evaluation of the probability that the mdr1-1Δ allele is segregating in the Elo population.

In **Chapter 4**, the haplotype analysis of the *MDR1* flanking region in the sample of Elo dogs is described.

Chapter 5 shows the search for functional polymorphisms in three exons of the canine *MDR1* gene.

Chapter 6 provides a general discussion and conclusions referring to Chapters 1-5.

Chapter 7 is a concise English summary of this thesis.

Chapter 8 is an expanded, detailed German summary which takes into consideration the overall research context.

Chapter 2

Review of prevalence, genetic aspects and adverse effects of the mdr1-1Δ mutation in dogs

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Deutsche Tierärztliche Wochenschrift

Review of prevalence, genetic aspects and adverse effects of the mdr1-1Δ mutation in dogs
Übersichtsartikel zu Prävalenz, genetischen Aspekten und negativen Auswirkungen der mdr1-1Δ-Mutation des Hundes

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Summary

A mutation in the canine *MDR1* gene causes multiple drug sensitivity in dog breeds of the Collie lineage. Dogs with this genetic defect show severe neurotoxic adverse effects if they are treated with particular drugs. Clinical signs depending on the administered drug and its concentration vary from mild toxicosis with salivation and disorientation to severe effects with coma and finally death of the dog. Drugs which provoke adverse effects are structurally different. Although they are used for many different indications, all of these drugs are substrates of a transporting protein encoded by the *MDR1* gene. This P-glycoprotein loses its normal protecting function at the tissue barriers in dogs with the mdr1-1Δ mutation. This article gives a short overview about the present state of analyses regarding the canine *MDR1* gene. The genetic background, effects and prevalence in affected dog breeds of the mdr1-1Δ mutation are summarized. On the one hand, the overview might help practical veterinarians to understand the aetiology of drug sensitivity in dogs with the mdr1-1Δ mutation, and on the other hand, it might point out appendages for future research works about the canine *MDR1* gene as well as for breeding strategies in affected dog breeds.

Keywords: *MDR1*, mutation, dog, drug sensitivity

Zusammenfassung

Eine Mutation im *MDR1*-Gen des Hundes verursacht bei Collies und den damit verwandten Hunderassen eine multiple Arzneimittelüberempfindlichkeit. Hunde mit diesem genetischen Defekt zeigen schwere neurotoxische Erscheinungen, wenn sie mit bestimmten Arzneimitteln behandelt werden. Klinische Symptome, die vom verabreichten Medikament und seiner Konzentration abhängen, variieren von milden Vergiftungserscheinungen mit vermehrtem Speichelfluss und Desorientiertheit bis zu starken Auswirkungen mit Koma und schließlich Tod des Tieres. Arzneimittel, die Nebenwirkungen verursachen, unterscheiden sich strukturell voneinander. Obwohl sie für viele verschiedene Indikationen benutzt werden, sind alle diese Arzneimittel Substrate des vom *MDR1*-Gen codierten P-Glykoproteins, das wichtige Transportfunktionen erfüllt. Dieses P-Glykoprotein verliert bei Hunden mit der mdr1-1Δ-Mutation seine normale Funktion an Gewebeschranken. Dieser Artikel gibt einen kurzen Überblick über den derzeitigen Stand der Forschung bezüglich des *MDR1*-Gens des Hundes. Der genetische Hintergrund, Auswirkungen und Vorkommen der mdr1-1Δ-Mutation in betroffenen Hunderassen werden zusammengefasst. Der Überblick soll einerseits praktizierenden Tierärzten helfen, die Ätiologie der Arzneimittelüberempfindlichkeit bei Hunden mit der mdr1-1Δ-Mutation zu verstehen, und andererseits Ansätze für zukünftige Forschungsarbeiten über das canine *MDR1*-Gen sowie für Zuchtstrategien in betroffenen Rassen aufzeigen.

Schlüsselworte: *MDR1*, Mutation, Hund, Arzneimittelüberempfindlichkeit

Introduction

The *MDR1* gene (*multidrug resistance* gene, also known as *ABCB1* gene, *ATP-binding cassette sub-family B member 1*) has been of particular interest for research work in humans and animals, i.e. mice and dogs, for several years now. The *MDR1* gene encodes the transmembrane protein pump P-glycoprotein. P-glycoprotein is considered to function as an ATP-driven membrane drug efflux pump and appears to play an important role in tumor cell resistance. P-glycoprotein was first described in Chinese hamster ovary (CHO) cells selected in culture for colchicine resistance (JULIANO and LING, 1976). In the 1980s the gene coding for P-glycoprotein was identified and named *multidrug resistance* (*MDR1*) gene, because of

its overexpression in multidrug resistant tumor cells (UEDA et al., 1987). P-glycoprotein is normally expressed in various mammalian tissues including brain capillary endothelial cells (CORDON-CARDO et al., 1989), the apical border of intestinal epithelial cells (LI et al., 1999), biliary canalicular cells (THIEBAUT et al., 1987), renal proximal tubular epithelial cells (HORI et al., 1993), placenta (LANKAS et al., 1998), and testes (MELAINE et al., 2002). P-glycoprotein actively extrudes selected xenobiotics from within the cell back into the lumen of brain capillary and intestine, whereas P-glycoprotein promotes excretion into the lumen of the bile canaliculi and renal tubules and therewith fulfils a twofold protective function.

The protecting function of P-glycoprotein in the blood-brain-barrier is to limit the passage resulting in lower concentrations of P-glycoprotein substrates in the brain tissue (SCHINKEL, 1998; FROMM, 2000). This fact was first described for *mdr1* knockout mice. SCHINKEL et al. (1994) found out that lack of P-glycoprotein leads to abnormally increased accumulation of certain drugs in the brain of these mice resulting in neurotoxic adverse effects.

A mutation in the canine *MDR1* gene causes drug sensitivity in several dog breeds. Affected dogs are inconspicuous until they have to be treated with a variety of drugs for different indications. In dogs with this mdr1-1Δ mutation severe neurotoxic adverse effects can be observed. Up to the present, the mutation was only found in presumably related dog breeds and traced back on a common origin.

The canine mdr1-1Δ mutation is associated with drug sensitivity

The first descriptions of ivermectin neurotoxicity in Collies were reported as early as 1983 (PRESTON, 1983; SEWARD, 1983). The observation that affected Collies had elevated concentrations of ivermectin in the central nervous system indicated that ivermectin neurotoxicity was caused by a defect in the blood-brain barrier (PULLIAM et al., 1985). For this phenomenon the designation ‘ivermectin-sensitive Collie’ was established in the literature (PAUL et al., 1987; TRANQUILLI et al., 1989).

The identification of a 4-bp deletion mutation in the *MDR1* gene of ivermectin-sensitive Collies as cause of the ivermectin neurotoxicity succeeded not until the year 2001 (MEALEY et al., 2001). A second research group could identify the same mutation independently (ROULET et al., 2003). The canine *MDR1* gene is located on *Canis familiaris* autosome 14

and composed of 28 exons. The detected mdr1-1Δ mutation in the fourth exon of this gene causes a frame-shift that generates a premature stop codon resulting in a severely truncated P-glycoprotein composed of < 10 % of the wild-type amino acid sequence. The truncated P-glycoprotein is presumably non-functional and could not be detected by Western blot analysis in ivermectin-sensitive Collies (ROULET et al., 2003).

A non-functional P-glycoprotein results in a loss of its protecting function. P-glycoprotein confers protection by limiting the uptake of compounds from the gastrointestinal tract and by contributing to their excretion via the liver, kidneys, and intestine. Moreover, P-glycoprotein in the blood-brain-barrier and other blood-tissue barriers protects sensitive organs from exposure to toxic compounds that may have entered the bloodstream (SCHINKEL, 1997). Accordingly, in dogs with the mdr1-1Δ mutation, the concentration of orally administered P-glycoprotein substrates in the bloodstream is increased because of augmented uptake from the gastrointestinal tract and reduced excretion via liver, kidney and intestine. Furthermore, the protecting function of blood-brain-barrier is largely lost so that P-glycoprotein substrates can easily entry through the membrane barrier into the central nervous system. They are not transported back into the brain capillary lumen, as it is normally the case, but accumulate in the brain tissue and thereby provoke neurotoxic adverse effects in affected dogs.

It has been demonstrated that the homozygous mdr1-1Δ/mdr1-1Δ genotype is strongly associated with the ivermectin-sensitive phenotype and an autosomal recessive inheritance pattern was evident (MEALEY et al., 2001).

Drug neurotoxicity in dogs with the mdr1-1Δ mutation

More than 50 therapeutic drugs are known substrates for human and murine P-glycoprotein. Because of the high degree of homology of P-glycoprotein between species, the same drugs are expected to be substrates of the canine P-glycoprotein (MEALEY et al., 2007). P-glycoprotein substrates include drugs for a diversity of indications as well as other xenobiotics. Table 1 shows a selection of P-glycoprotein substrate drugs with veterinary usage that may cause neurotoxic adverse effects in dogs with an mdr1-1Δ allele (reviewed in MEALEY, 2004 and GEYER et al., 2005b). Studies specially addressing this question are rare. In addition to ivermectin, neurotoxic adverse effects in Collies or in other mdr1-1Δ mutant dogs were described for doramectin (YAS-NATAN et al., 2003; GEYER et al., 2007),

loperamide (SARTOR et al., 2004), digoxin (HENIK et al., 2006), moxidectin (GEYER et al., 2005a) and dexamethasone (MEALEY et al., 2007). The P-glycoprotein substrate drugs vincristine, vinblastine and doxorubicin provoked adverse effects in a heterozygous MDR1/mdr1-1Δ Collie (MEALEY et al., 2003).
The permeability of the blood-brain-barrier caused by a nonfuctional P-glycoprotein results in significantly higher concentrations of the P-glycoprotein substrates in the brain tissue. Typically, the concentration of ivermectin in brain tissue of Beagles is between 10 and 100 times lower than concentrations in plasma and liver, respectively. In contrast, in ivermectin-sensitive Collies, the concentration of ivermectin in brain tissue exceeds both liver and plasma ivermectin concentrations (PULLIAM et al., 1985). Ivermectin-sensitive Collies show neurotoxic signs when exposed to a single dose of 0.1 - 0.2 mg/kg body weight ivermectin orally whereas a dosage of 2.5 mg/kg body weight can be administered in nonsensitive Collies and Beagles without signs of toxicosis (PULLIAM et al., 1985; PAUL et al., 1987).
Accumulation in the brain tissue is dose-dependent and causes neurotoxic signs as mydriasis, salivation, somnolence, disorientation, confusion, depression, ataxia, tremors, nonresponsiveness, coma and death (PAUL et al., 1987; TRANQUILLI et al., 1989).

Effects of the mdr1-1Δ mutation in other organs and on hormone release

P-glycoprotein is normally involved in limiting the absorption of orally administered drugs in the intestine, in promoting the elimination of drugs in liver, kidney and intestine as well as in restricting drug entry into the central nervous system, the testis and the placenta from the bloodstream (SCHINKEL, 1997). Administering P-glycoprotein substrate drugs to dogs with the mdr1-1Δ mutation may provoke negative effects on the drug transport and cause toxicity in the expression organs and in the foetus.
Recently, MEALEY et al. (2007) performed a study concerning the comparison of the hypothalamic-pituitary-adrenal (HPA) axis in mdr1-1Δ mutant and MDR1 wild-type dogs. Because of significant differences in the measurement of basal plasma cortisol concentrations, cortisol concentrations after ACTH (adrenocorticotropin hormone) stimulation and plasma ACTH concentrations after dexamethasone administration between mdr1-1Δ mutant and MDR1 wild-type dogs, it was suggested that P-glycoprotein plays a role in the regulation of

the HPA axis. Furthermore, it appears that lack of functional P-glycoprotein causes a suppression of the HPA axis in mdr1-1Δ mutant dogs compared to MDR1 wild-type dogs.

Prevalence of the mdr1-1Δ mutation in different dog breeds

In the last years, several studies were performed concerning the prevalence of the canine mdr1-1Δ mutation in different dog breeds. Mainly, British herding dog breeds from the Collie lineage in different countries were of particular interest. Frequencies of the mdr1-1Δ mutation in Collies and related dog breeds from the northwestern United States (MEALEY et al., 2002), the entire USA (NEFF et al., 2004), France (HUGNET et al., 2004), Germany (GEYER et al., 2005b), Japan (KAWABATA et al., 2005) and Australia (MEALEY et al., 2005) were determined. Table 2 gives an overview on reported prevalences of the mdr1-1Δ mutation in affected dog breeds from different countries.

Additionally, there are few further studies concerning miscellaneous dog breeds. The broadest analysis was performed by NEFF et al. (2004). In addition to the breeds from the Collie lineage, the research group screened a multibreed panel with > 90 different dog breeds of varying origin. There were several European herding dog breeds such as the German Shepherd or Belgian Malinois, several sighthound breeds such as the Greyhound or the Whippet and a diversity of other dog breeds such as the Labrador Retriever or the Bernese Mountain Dog. Thereby, the mdr1-1Δ mutation was also found in two breeds from the sighthound class, the Longhaired Whippet and the Silken Windhound, for which a relation to the Collie lineage was supposed. In the other dog breeds of the multibreed panel, no mdr1-1Δ mutation could be detected although the number of animals was not sufficient in many cases to exclude the prevalence of the mutation for the entire breed.

In a study from Japan, the mutant allele could not be detected in the dog breeds Labrador Retriever, Golden Retriever, Shih Tzu, Shiba Inu and Dachshund which were analysed in addition to the known affected breeds Collie, Australian Shepherd and Shetland Sheepdog (KAWABATA et al., 2005).

In a recently performed study, FECHT et al. (2007a) could exclude the prevalence of the mdr1-1Δ mutation for the newly developed German dog breed Elo that was founded amongst others by the affected Old English Sheepdog breed. The Old English Sheepdog breed

contributed 20.79 % of the genes to the current Elo population and thus, Elos were suspected to carry the mdr1-1Δ mutation.

To evaluate the origin of the mdr1-1Δ allele and the *MDR1* flanking region, a haplotype analysis was designed. Therefore, four microsatellite markers flanking the *MDR1* gene were used. NEFF et al. (2004) found that the mdr1-1Δ allele was identical by descent among affected dog breeds as evidenced by a single ancestral haplotype. Based on breed histories and the extent of linkage disequilibrium, it was concluded that all dogs carrying the mdr1-1Δ allele are descendants of a dog that lived in Great Britain before the genetic isolation of breeds by registered breeding. FECHT et al. (2007b) employed a haplotype analysis based on the same four microsatellite markers to exclude that the Elo breed was still at risk for the mdr1-1Δ mutation by testing for the presence of the Collie lineage haplotype. In this study, the identical *MDR1* flanking region which had previously been associated with the mdr1-1Δ allele in Old English Sheepdogs (NEFF et al., 2004) was detected in Elos. However, this haplotype did not include the mdr1-1Δ mutation and could be traced back to the Japanese Spitz as one of the founder dog breeds of the Elo.

In White Swiss Shepherd dogs, the mdr1-1Δ allele could be detected in a frequency of 13 % and in addition, two cases of doramectin toxicosis from this dog breed could be associated with the mdr1-1Δ mutation (GEYER et al., 2007). Carriers of the mdr1-1Δ allele in White Swiss Shepherd dogs shared the haplotype of the *MDR1* region with sighthounds and thus, the origin of the mdr1-1Δ mutation was assumed from the sighthound lineage and inbreeding may have led to mdr1-1Δ homozygous animals (GEYER et al., 2007).

Effects on dog breeding

Genotype-based selection can eliminate the mutation from the gene pool of affected dog breeds in a few generations in dependence of the mdr1-1Δ allele frequency. The design of breeding strategies has to regard the mdr1-1Δ allele frequency in the particular dog breed and the population size because the effective population size is reduced and inbreeding is increased with the proportion of heterozygote carriers excluded from breeding (Table 3). The effective population size (Ne), increase of inbreeding per generation (ΔF) and ΔF per 10 generations (ΔF-10) were calculated using the formulas: $Ne = 4 \times M \times W/(M + W)$, $\Delta F = 1/(2Ne)$ and $\Delta F\text{-}10 = 1 - (1- \Delta F)^{10}$, with M = number of male breeding animals, W = number

of female breeding animals. The reduction of Ne through selection for the MDR1 allele was computed according to the number of heterozygous mdr1-1Δ animals allowed for breeding. In the case of low mdr1-1Δ allele frequencies (0.1 - 0.3), most of the heterozygote carriers can be banned from breeding without large increases of inbreeding rates per generation. By contrast, when the mdr1-1Δ allele frequencies are larger than 0.3, specific measures have to be taken to avoid increases of inbreeding coefficients larger than 2 % per generation. For example in a small population of 100 females and 10 males, the increase of inbreeding rate per generation is enormous with 10.42 % if the mdr1-1Δ allele frequency is 0.7 and 90 % of the heterozygous carriers should be excluded from breeding. It has to be considered that enough sires are available for breeding in relation to the number of dams. Enlargement of the population size by increasing the number of dams does not effectively result in lower inbreeding rates. The number of matings per sire has to be limited and the percentage of heterozygote by homozygote matings (MDR1/mdr1-1Δ x MDR1/MDR1) has to be increased for a limited number of generations. It can be concluded that populations with high mdr1-1Δ allele frequencies of about 50 to 60 % like the Collie breed have to elaborate individual breeding strategies depending on the number of dams and sires available for breeding. These dog breeds can not immediately exclude most of the heterozygous carriers in addition to the homozygous carriers which should be generally banned from breeding otherwise the increase of inbreeding rate is huge. Therefore, the result of gene test for the mdr1-1Δ allele should be taken into account when puppies are pre-selected for breeding. Employing the gene test at this early stage is useful to reduce the mdr1-1Δ allele in the candidates of later breeding animals.
A further aspect has to be considered when elimination of the mdr1-1Δ mutation is performed. In previous studies, four microsatellite markers with positions between 15.6 and 19.3 Mb on dog chromosome 14 were used to analyse the *MDR1* flanking region in different dog breeds (NEFF et al., 2004; FECHT et al. 2007b; GEYER et al., 2007). A haplotype containing the mdr1-1Δ mutation was conserved among affected breeds of the Collie lineage indicating that loci adjacent to the *MDR1* gene are jointly inherited due to rare recombinations in small genomic segments. In a region of 2 Mb surrounding the *MDR1* gene, there are 14 other genes located. Due to this fact, alleles with unfavourable effects that are in coupling phase with the MDR1 allele (wild type allele) for which dogs are selected can be rapidly increase (Table 4). This can result in an unforeseen selection response for undesired traits and

has to be taken into consideration for breeding strategies. The frequency of the undesired allele B was derived using a two-locus model, two different mating types, different mdr1-1Δ allele frequencies and recombination rates of 0.01, 0.1 and 0.3 between the *MDR1* gene and a second locus with alleles A and B as given in Table 4. The genotype and allele frequencies of the undesired allele B in the next generation are dependent of the mdr1-1Δ allele frequency ($f_{mdr1\text{-}1\Delta}$), the recombination rate (r) and the mating type. The gamete frequencies can be calculated using $f_{mdr1\text{-}1\Delta}$ x r, $f_{mdr1\text{-}1\Delta}$ x (1-r), f_{MDR1} x r and f_{MDR1} x (1-r) for the joint frequency of the alleles mdr1-1Δ and B in repulsion phase, mdr1-1Δ and A in coupling phase, MDR1 and A in repulsion phase and MDR1 and B in coupling phase. After multiplying the gamete frequencies according to the respective mating types, the genotype and allele frequencies in the next generation can be deduced. The frequency of the A allele was assumed identical with the frequency of the mdr1-1Δ allele in the base generation and correspondingly the same relation between the MDR1 and B allele was assumed. Particularly, when the frequency of the mdr1-1Δ allele is high and there is a negative allele (B) completely coupled with the MDR1 allele, frequencies of this undesired allele dramatically increase even if recombination is as large as 0.3. This means also allelic effects linked with the MDR1 allele in a large distance of about 30 to 40 Mb (30-40 cM) proximally and distally of the *MDR1* gene may be seen in MDR1 selected dogs. Furthermore, when only dogs are allowed to breed which are homozygous for the MDR1 allele, the closely linked alleles in repulsion phase with the MDR1 allele may also disappear. Thus, a breeding strategy that strictly bans all homozygote and heterozygote carriers of the mdr1-1Δ allele when this allele has a high frequency likewise in Collies can not be recommended due to unknown effects of alleles from closely linked loci. Studies investigating the associations with other breeding traits have to be performed before a selection strategy for elimination of the mdr1-1Δ mutation can be established for the respective dog breeds.

Conclusions

The mdr1-1Δ mutation is of large relevance for several dog breeds. Breeding strategies should aim to confine or eliminate the mdr1-1Δ allele. The progress towards mdr1-1Δ allele free populations is strongly depending on its prevalence, the population size and possible correlated side-effects of the MDR1 allele and thus has to be planned carefully.

Anyhow, there is a great hazard for the individual dog with the mdr1-1Δ mutation in the case of required drug therapy. To identify individuals that might be predisposed to adverse drug effects, veterinarians can use DNA testing of mdr1-1Δ to screen animals before administering certain therapeutic drugs. DNA tests are offered by several commercial laboratories, so it is not necessary to exclude individuals or entire breeds from an effective treatment. Furthermore, negative effects on the physical constitution of dogs with the mdr1-1Δ mutation, like the effect on the HPA axis, are only partly known up to the present. Therefore, further studies concerning the canine *MDR1* gene are urgently warranted.

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Tab. 1: **Selected P-glycoprotein substrates with usage in veterinary medicine. Drugs with documented neurotoxicity in dogs with the mdr1-1 mutation are in bold.**

Antiparasitic agents	**Ivermectin** **Doramectin** **Moxidectin**
Antimicrobial agents	Erythromycin Ketoconazole Tetreacycline Doxycycline Levofloxacin
Anticancer agents	**Doxorubicin** **Vincristine** **Vinblastine** Docetaxel Paclitaxel
Cardiac drugs	**Digoxin** Verapamil Diltiazem Talinolol
Opioids	**Loperamide** Morphine
Steroid hormones	**Dexamethasone** Aldosterone Cortisol Methylprednisolone
Immunosuppressants	Cyclosporine A Tacrolimus
Antiemetic drugs	Ondansetron Domperidon

Tab. 2: **Frequencies of the mdr1-1Δ mutation in affected dog breeds from different countries**

Country	Dog breed	n	mdr1-1Δ (%)	MDR1/ MDR1 (%)	MDR1/ mdr1-1Δ (%)	mdr1-1Δ/ mdr1-1Δ (%)	Reference
Germany	Collie	578	54.6	23.9	43.1	33.0	GEYER et al., 2005b
	Shetland Sheepdog	140	30.0	45.7	48.6	5.7	
	Australian Shepherd	333	19.5	67.9	25.2	6.9	
	Wäller	62	18.5	62.9	37.1	0.0	
	Old English Sheepdog	24	6.3	87.5	12.5	0.0	
	Border Collie	334	0.6	99.1	0.6	0.3	
	Bearded Collie	29	0.0	100.0	0.0	0.0	
France	Collie	25	64.0	20.0	32.0	48.0	HUGNET et al., 2004
USA	Australian Shepherd	178	16.6	68.5	29.8	1.7	NEFF et al., 2004
	Mini. Austr. Shepherd	56	25.9	51.8	44.6	3.6	
	Collie	263	54.6	22.0	46.8	31.2	
	Old English Sheepdog	151	3.6	92.7	7.3	0.0	
	Shetland Sheepdog	190	8.4	84.2	14.7	1.1	
	McNab	35	17.1	68.6	28.6	2.8	
	English Shepherd	91	7.1	85.7	14.3	0.0	
	Longhaired Whippet	89	41.6	32.6	51.7	15.7	
	Silken Windhound	84	17.9	65.5	33.3	1.2	

Tab. 2: **continued**

Country	Dog breed	n	mdr1-1Δ (%)	MDR1/ MDR1 (%)	MDR1/ mdr1-1Δ (%)	mdr1-1Δ/ mdr1-1Δ (%)	Reference
USA-northwest	Collie	40	56.0	22.0	42.0	35.0	MEALEY et al., 2002
Australia	Collie	33	56.0	12.0	64.0	24.0	MEALEY et al., 2005
	Australian Shepherd	14	42.5	36.0	43.0	21.0	
	Shetland Sheepdog	7	21.5	57.0	43.0	0.0	
	Border Collie	7	0.0	100.0	0.0	0.0	
Japan	Collie	12	58.3	25.0	33.3	41.7	KAWABATA et al., 2005
	Australian Shepherd	9	33.3	44.4	44.4	11.1	
	Shetland Sheepdog	42	1.2	97.6	2.4	0.0	

Tab. 3: **Effective population size and increase of inbreeding rate per generation (%) in dependency of the frequency of the mdr1-1Δ mutation, selection intensity of heterozygote carriers and population size. Homozygote carriers are generally banned from breeding and all these dogs are assumed to be known in this calculation.**

Frequency of the mdr1-1Δ mutation	Proportion of heterozygote carriers excluded from breeding	Effective population size	Increase of inbreeding rate (%) per	
			1 generation	10 generations
Population size: 100 females, 20 males				
0.1	0.0	66	0.76	7.3
	0.5	60	0.83	8.0
	0.9	55	0.91	8.7
0.3	0.0	61	0.82	7.9
	0.5	47	1.07	10.2
	0.9	35	1.41	13.2
0.5	0.0	50	1.00	9.6
	0.5	33	1.50	14.0
	0.9	20	2.50	22.4
0.7	0.0	34	1.47	13.8
	0.5	20	2.50	22.4
	0.9	9	5.68	44.3
Population size: 100 females, 10 males				
0.1	0.0	36	1.39	13.1
	0.5	33	1.53	14.3
	0.9	30	1.66	15.4
0.3	0.0	33	1.51	14.1
	0.5	26	1.96	18.0
	0.9	19	2.59	23.0
0.5	0.0	27	1.83	16.9
	0.5	18	2.75	24.0
	0.9	11	4.58	37.5
0.7	0.0	19	2.70	23.9
	0.5	11	4.58	37.5
	0.9	5	10.42	66.7

Tab. 3: **continued**

Frequency of the mdr1-1Δ mutation	Proportion of heterozygote carriers excluded from breeding	Effective population size	Increase of inbreeding rate (%) per	
			1 generation	10 generations
Population size: 500 females, 10 males				
0.1	0.0	38	1.29	12.2
	0.5	35	1.42	13.3
	0.9	32	1.54	14.4
0.3	0.0	36	1.40	13.2
	0.5	27	1.82	16.8
	0.9	21	2.40	21.5
0.5	0.0	29	1.70	15.8
	0.5	20	2.55	22.8
	0.9	12	4.25	35.2
0.7	0.0	20	2.50	22.4
	0.5	12	4.25	35.2
	0.9	5	9.66	63.8
Population size: 1000 females, 10 males				
0.1	0.0	39	1.28	12.0
	0.5	36	1.40	13.2
	0.9	33	1.52	14.2
0.3	0.0	36	1.39	13.0
	0.5	28	1.80	16.6
	0.9	21	2.37	21.4
0.5	0.0	30	1.68	15.6
	0.5	20	2.53	22.6
	0.9	12	4.21	34.9
0.7	0.0	20	2.48	22.2
	0.5	12	4.21	34.9
	0.9	5	9.56	63.4

Tab. 4: **Effect of selection against the mdr1-1Δ mutation in the first generation on the frequency of an allele in coupling phase with the MDR1 allele located on a linked locus when only individuals with the genotype MDR1/MDR1 and/or matings among MDR1/mdr1-1Δ and MDR1/MDR1 individuals are allowed for breeding (Mating types: Hom x Hom = MDR1/MDR1 x MDR1/MDR1, Hom x Het = MDR1/MDR1 x MDR1/mdr1-1Δ).**

Frequency of the mdr1-1Δ and the B allele in coupling phase with the MDR1 allele	Proportion of heterozygote carriers prevented from breeding	Recombination rate between the MDR1 locus and a linked locus	Frequency of the allele B which is in coupling phase with the MDR1 allele dogs are selected for		
			Mating type		
			Hom x Hom	Hom x Het	0.5 Hom x Hom + 0.5 Hom x Het
arbitrary	1.0	0.01	0.99	-	-
	1.0	0.10	0.90	-	-
	1.0	0.30	0.70	-	-
0.1/0.9	0.0	0.01	-	0.90	0.95
	0.0	0.10	-	0.79	0.84
	0.0	0.30	-	0.58	0.62
0.1/0.9	0.5	0.01	-	0.94	0.96
	0.5	0.10	-	0.82	0.86
	0.5	0.30	-	0.59	0.62
0.1/0.9	0.9	0.01	-	0.97	0.98
	0.9	0.10	-	0.85	0.87
	0.9	0.30	-	0.59	0.62
0.7/0.3	0.0	0.01	-	0.67	0.83
	0.0	0.10	-	0.62	0.76
	0.0	0.30	-	0.53	0.59
0.7/0.3	0.5	0.01	-	0.70	0.85
	0.5	0.10	-	0.65	0.77
	0.5	0.30	-	0.54	0.60
07./0.3	0.9	0.01	-	0.84	0.92
	0.9	0.10	-	0.75	0.82
	0.9	0.30	-	0.57	0.61

Chapter 3

Analysis of the canine mdr1-1Δ mutation in the dog breed Elo

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Analysis of the canine mdr1-1Δ mutation in the dog breed Elo

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Summary

A deletion mutation in the canine multidrug resistance gene, *MDR1*, is associated with drug sensitivity. This was shown for several pure bred dog breeds from the Collie lineage such as the Collie (rough-coated and smooth-coated), the Australian Shepherd and the Old English Sheepdog. To determine whether the mdr1-1Δ mutation could be found in the newly bred German dog breed Elo which is based amongst other breeds on Old English Sheepdogs, 177 blood samples representative for the Elo breed were collected. After DNA extraction, a polymerase chain reaction (PCR) based method with subsequent polyacrylamide gel electrophoresis was used for detection of the mdr1-1Δ mutation. The mdr1-1Δ allele was not observed in the Elos investigated. The probability that the mdr1-1Δ allele which originated in the Old English Sheepdog breed is segregating in the Elo population, was estimated at 3.68 x 10^{-17}.

Introduction

A mutation in the canine multidrug resistance gene, *MDR1* (*ABCB1*, ATP-binding cassette sub-family B member 1), causes drug sensitivity with neurotoxic signs in several dog breeds from the Collie lineage. This phenomenon is called ivermectin sensitivity because it was first described for the drug ivermectin in Collies in 1983 (Preston, 1983; Seward, 1983). Neurotoxicity is provoked in some, but not all Collies by doses that are a fraction of what is required for other dogs. Ivermectin-sensitive Collies show neurotoxic signs when exposed to 0.12 – 0.15 mg/kg body weight ivermectin orally whereas a dosage of 2.5 mg/kg body weight can be administered in ivermectin-nonsensitive Collies and Beagles without signs of toxicosis (Pulliam et al., 1985; Paul et al., 1987; Tranquilli et al., 1989). The canine *MDR1* gene is

located on *Canis familiaris* chromosome 14 and composed of 28 exons. The cause of the susceptibility is a 4-bp deletion mutation in the fourth exon of the *MDR1* gene which causes a frame-shift accompanied by multiple premature stop codons resulting in a severely truncated P-glycoprotein composed of < 10% of the wild-type amino acid sequence (Mealey et al., 2001; Roulet et al., 2003). P-glycoprotein, the product of the *MDR1* gene, is an ATP-dependent drug transporter that is expressed on the luminal membrane of brain capillary endothelial cells and plays an important role in the blood-brain barrier. Moreover, P-glycoprotein is expressed in other organs such as the liver, kidney, intestine, testis and placenta (Cordon-Cardo et al., 1989, 1990; Fromm, 2000, 2004). The function of P-glycoprotein in the blood-brain barrier is to extrude a variety of substrates, including ivermectin, from brain tissue back into the capillary endothelial cells resulting in lower concentrations of these substrates in the brain (Schinkel, 1998; Fromm, 2000). In mdr1 knockout mice, lack of P-glycoprotein leads to abnormally increased accumulation of certain drugs in the brain resulting in neurotoxic adverse effects (Schinkel, 1998). Thus, the mutant mdr1-1Δ allele results in truncated P-glycoprotein with probably a complete loss of its protecting function although this fact has not yet been established (Roulet et al., 2003). Accordingly, the P-glycoprotein-substrates penetrate the brain tissue and provoke neurotoxic adverse effects in dogs homozygous for the mdr1-1Δ mutation. It has been demonstrated that the homozygous mdr1-1Δ/mdr1-1Δ genotype is strongly associated with the ivermectin-sensitive phenotype (Mealey et al., 2001). Several other P-glycoprotein-substrate drugs used in the treatment of dogs may provoke neurotoxic adverse effects if there is an mdr1-1Δ allele. The P-glycoprotein-substrate drugs vincristine, vinblastine and doxorubicin provoked adverse effects in a heterozygous MDR1/mdr1-1Δ Collie (Mealey et al., 2003). Moreover, neurotoxic adverse effects were described for several other drugs in Collies or in mdr1-1Δ mutant dogs. These included doramectin (Yas-Natan et al., 2003), loperamide (Sartor et al., 2004), digoxin (Henik et al., 2006), moxidectin (Geyer et al., 2005b) and dexamethasone (Mealey et al., 2007). Accumulation in the brain depends on the concentration of the administered drug and causes signs as mydriasis, salivation, somnolence, depression, disorientation, ataxia, tremors, coma and death (Paul et al., 1987; Tranquilli et al., 1989).

It was shown that the mdr1-1Δ allele is identical by descent and the mutation probably originated in a working sheepdog in Great Britain before the emergence of breeds. From

there, the mdr1-1Δ allele was dispersed over several British herding dog breeds from the Collie lineage such as rough-coated and smooth-coated Collie, Australian Shepherd or Shetland Sheepdog (Neff et al., 2004).

One of these breeds is the Old English Sheepdog which carries the mdr1-1Δ allele. Neff et al. (2004) observed an mdr1-1Δ allele frequency of 3.6% in a sample of 151 Old English Sheepdogs and Geyer et al. (2005a) determined an allele frequency of 6.3% in 24 Old English Sheepdogs. In both studies no homozygous mdr1-1Δ/mdr1-1Δ genotype could be discovered due to the low allele frequency. The dog breeds of the Collie lineage share the same ancestors which were working sheepdogs in Great Britain before the emergence of pure bred dog breeds. The Old English Sheepdog is believed to be genetically isolated from the other Collie-related breeds since the foundation of the Kennel Club of England in 1873 when the pure breeding began (Neff et al., 2004).

The Elo is a newly developed dog breed. The breeding of the Elo was started in 1987 in Hannover, Germany, on the basis of 16 founder animals stemming from nine different dog breeds as following: Eurasian Dog, Old English Sheepdog, Chow-Chow, Samoyed, Dalmatian, Pekingese, Pomeranian Dog, German Spitz and Japanese Spitz.

There were four dogs of the breed Old English Sheepdog which participated in the foundation of the Elo. The most important Old English Sheepdog was a female dog which littered the first crossbreed puppies. Furthermore, her pure bred Old English Sheepdog son and another pure bred male dog became founders. The fourth dog which contributed Old English Sheepdog genes was a female crossbreed of Old English Sheepdog x Eurasian Dog and Chow-Chow (Szobries and Szobries, 2004).

The Old English Sheepdog breed contributed about 23 % of the genes to the whole Elo population (Kaufhold et al., 2005).

The aim of this study was to determine whether the mutant mdr1-1Δ allele with an origin in the Old English Sheepdog breed is segregating in the Elo population.

Materials and Methods

Sample ascertainment and classification

Blood samples from 177 Elos were collected. As reference three Collies previously genotyped for the mdr1-1Δ mutation and comprising all three genotypes were available.

Pedigree data for the Elo population included all dogs from the beginning of breeding in 1987 until the end of the year 2005.

The analysis of the pedigree data was carried out using the programme OPTI-MATE, version 3.87 (Wrede and Schmidt, 2003) to calculate gene contributions by the different founder animals. The procedures FREQ and MEANS of SAS, version 9.1.3 (Statistical Analysis System, SAS Institute, Cary, NC, USA) were employed for further statistical evaluations of these gene contributions.

Dogs were randomly drawn from the current Elo population, which had to be defined by the dogs born from 1994 until 2005 because information about death of individuals was not reliably reported.

Gene contributions by the four Old English Sheepdog founders were calculated for the Elos of the sample as well as for the dogs of the current Elo population and subdivided in ten classes to give an overview about the importance and distribution of the Old English Sheepdog founder dogs in the Elo population (Table 1).

The pure bred female Old English Sheepdog (Dam A), the pure bred male Old English Sheepdog (Sire C) and the female Old English Sheepdog crossbreed (Dam D) are known founder animals in the pedigree data of the Elo population. We created an independent phantom sire (Sire B) for the pure bred Old English Sheepdog son as fourth founder animal to allow for the comparison between the gene contributions of each individual founder Old English Sheepdog. It was approved that the phantom sire was a pure bred Old English Sheepdog but further pedigree was unknown.

For calculation of the gene contributions, the descent from the four Old English Sheepdog founders was decisive. The gene contributions describe the proportion of genes (in %) of each Old English Sheepdog founder (A, B, C, D) for the respective Elo of the sample (n_s) and of the current Elo population (n_p). Additionally, the gene contributions by the four Old English Sheepdog founders in total (OES Founders) to each Elo were calculated based only on breed affiliation.

The distribution of the gene contributions in the sample was aligned with the distribution in the current population to obtain a representative cross-section of the Elo population.
Mean values were calculated for the proportion of genes contributed by the four Old English Sheepdog founders to the dogs of the sample and to the current Elo population. The four Old English Sheepdog founders in total contributed 20.79% (range: 0 – 57.04%) of the genes to the current Elo population and 18.76% to the dogs of the sample.
Dam A had the most influence on the Elo population. Only 31 of 2973 dogs of the current Elo population were not related to Dam A. The contribution of this female dog to the gene pool of the Elos ranged from 0% to 40% whereas the three other Old English Sheepdog founders ranged mainly in lower proportions. The mean value of gene contribution by Dam A was 16.31% in the sample and 17.42% in the current population. It followed that each of the other three Old English Sheepdog founders only contributed a marginal proportion of genes to the current Elo population which ranged each lower than 2%.

Genotyping
Genomic DNA was isolated from EDTA (ethylenediaminetetraacetic)-anticoagulated blood using the QIAamp® 96 Spin Blood Kit (Qiagen, Hilden, Germany). For amplification, recently published primer sequences were used (Hugnet et al., 2004). The primer sequences were as follows: forward primer (MDR1_F700) 5'-GGC TTG ATA GGT TGT ATA TGT TGG TG-3' and reverse primer (MDR1_R) 5'-ATT ATA ACT GGA AAA GTT TTG TTT-3'. The forward primer was 5'-endlabeled with IRD700 fluorescent dye. The primers (MWG-Biotech, Ebersberg, Germany) spanned the mdr1-1Δ mutation in the canine *MDR1* gene generating 148 bp and 144 bp products for the wild-type and the mutant allele, respectively. PTC 100™ or PTC 200™ (MJ Research, Watertown, MA, USA) thermocyclers and a general PCR programme with optimum annealing temperature (Ta) of 55 °C were used for the PCR amplification. The reaction started with denaturation at 94 °C for 4 min, followed by 32 cycles comprising denaturation at 94 °C for 30 sec, annealing at T_a (55 °C) for 1 min, and extension at 72 °C for 30 sec. The PCR was completed with a final cooling at 4 °C for 10 min. PCR reaction was performed in 12 µl reaction volumes using 2 µl (~ 20 ng/µl) genomic DNA, 1.2 µl 10x PCR buffer, 0.3 µl DMSO, 0.6 µl (10 µM) of each primer, 0.12 µl dNTPs (10mM each) and 0.2 µl (5 U/µl) *Taq* Polymerase (Qbiogene, Heidelberg, Germany). For the

analysis of the genotypes, the PCR products were size-fractionated by gel electrophoresis on an automated sequencer (LI-COR, Lincoln, NE, USA) using 6% polyacrylamide denaturing gels (RotiphoreseGel40, Carl Roth, Karlsruhe). Allele sizes were detected using an IRD700-labelled DNA ladder, and the genotypes were assigned by visual examination.
The probability was estimated that there is no mdr1-1Δ allele segregating in the current Elo population based on the 177 genotyped animals.

Results

We genotyped the *MDR1* gene for the mdr1-1Δ mutation in 177 Elos. Only PCR fragments with lengths of 148 bp could be detected, thus all Elos were homozygous for the wild-type MDR1 allele. Figure 1 shows the results for three Elos of the sample. To discriminate the homozygous MDR1/MDR1 genotype from the heterozygous MDR1/mdr1-1Δ genotype and the homozygous mdr1-1Δ/mdr1-1Δ genotype, three previously tested Collies with different genotypes were used as reference.
The probability (p) that no mdr1-1Δ allele with an Old English Sheepdog origin is segregating in the Elo breed, was calculated on the following assumptions: The four Old English Sheepdog founders in the Elo breed were randomly drawn animals from the Old English Sheepdog population. The mdr1-1Δ allele frequency in this population was at 0.1 and in Hardy-Weinberg equilibrium. The probability that at least one of the four founders was heterozygous was:
$p_1 = 1 - (1 - 0.1)^4 = 0.3439$.
Assuming at least one of the four Old English Sheepdog founders was heterozygous for the mdr1-1Δ locus, the probability to observe at least one heterozygous offspring in the sample was calculated by using a binomial function with the average gene contribution by the four founders and the sample size as parameters:
$p_2 = (1 - gc_{A.S})^n = 1.07 \times 10^{-16}$,
with n = 177, number of dogs genotyped; $gc_{A.S} = 0.1876$, average gene contribution by Old English Sheepdog founder animals in the sample genotyped.
The probability (p_2) that no heterozygous offspring of the founders should be observed, given at least one founder was heterozygous, was multiplied with the probability (p_1) that at least

one founder was heterozygous. The probability (p) that no mdr1-1Δ allele originated in the Old English Sheepdog breed is segregating in the Elo population is then as follows:
$p = 1 - p_2 \times p_1 = 1 - 3.68 \times 10^{-17} \approx 1$.

Discussion

The occurrence of a 4-bp deletion in the canine *MDR1* gene which causes drug sensitivity was demonstrated for Collies and related dog breeds. In several studies the frequencies of the mutant mdr1-1Δ allele were determined in the British herding dog breeds Collie, Old English Sheepdog, English Shepherd, McNab, Border Collie, Australian Shepherd and Shetland Sheepdog (Neff et al., 2004; Geyer et al., 2005a; Kawabata et al., 2005; Mealey et al., 2005). Additionally, Neff et al. (2004) found the mutation in the sighthound breeds Longhaired Whippet and Silken Windhound. The Longhaired Whippet is described as an ancient variety of sighthound that was restored in the 1950s by a single breeder who also bred Shetland Sheepdogs. The Longhaired Whippet and the Shetland Sheepdog favour the same haplotype so it was speculated an introgression of the Shetland Sheepdog (Neff et al., 2004). The Silken Windhound was developed more recently by crossing multiple sighthound breeds including the Longhaired Whippet (Neff et al., 2004). Geyer et al. (2005a) discovered the mdr1-1Δ allele in the newly developed dog breed Wäller, a crossing of Briards and Australian Shepherds. The two sighthound breeds and the Wäller have in common to be developed by crossings of British herding dogs carrying the mutant mdr1-1Δ allele. The mutation could not yet be found in other dog breeds, e.g. in the founder breeds of the Elo Chow-Chow, Samoyed, Dalmatian and Pomeranian Dog whereas no broad screening of these breeds was made (Neff et al., 2004; Kawabata et al., 2005). Because of the conclusions that the mdr1-1Δ allele is identical by descent and that the mutation originated in a sheepdog in Great Britain before breeding by registry in 1873 (Neff et al., 2004), the mutant mdr1-1Δ allele is presumably restricted to British herding dogs and related breeds.
One of the affected breeds, the Old English Sheepdog, was an important founder breed of the newly developed dog breed, the Elo. The large gene contribution by the Old English Sheepdog to the Elo breed led to the assumption that the mdr1-1Δ allele might also be prevalent in the Elo population. It was unlikely that the mdr1-1Δ allele might have been

originated in any of the other founder breeds. Although no homozygously mutated mdr1-1Δ/mdr1-1Δ genotype was analysed in Old English Sheepdogs and the allele frequency was low (Neff et al., 2004; Geyer et al., 2005a), the possibility was given that the mutant mdr1-1Δ allele segregated in the Elo population as it was the case in other crossed dog breeds, e. g. the Silken Windhound and the Wäller.

Therefore, a representative sample of 177 Elos was analysed for the mdr1-1Δ mutation. All tested Elos were homozygous for the wild-type MDR1 allele.

The Elos of the sample represented each of the four Old English Sheepdog founders. The distribution of the proportions of Old English Sheepdog genes in the sampled Elos should resemble those in the current Elo population to obtain a representative cross-section of the population.

The mean values of the Old English Sheepdog gene contributions were lower in the sample than in the current population although the sample should resemble the current population structure. This was obvious for each of the four founder animals as well as for the gene contribution by the four founders in total. The mean value of the gene contribution in total was 18.76% in the sampled Elos and 20.79% in the current Elo population. The lower gene contributions could be explained with an ongoing change of the population structure in the recent years. The Old English Sheepdog gene contribution has decreased in the Elo population from the beginning of breeding up to the present whereas the Eurasian gene contribution remained constant (Kaufhold et al., 2005). The predominant fraction of dogs of the sample consisted of Elos born from 2000 until 2005 which was reflected in lower gene contributions by the Old English Sheepdog. Consequently, a lower gene contribution in the sample than in the current population could hardly be avoided, particularly because fewer older dogs born from 1994 until 1999 were available for the collection of blood samples.

We estimated a probability of close to one that no mdr1-1Δ allele with an Old English Sheepdog origin is segregating in the Elo population.

Because of the representative sample and the estimated probability, it is very unlikely that the mutant mdr1-1Δ allele still occurs in the current Elo population. Therefore, no consequences arise from the results of this study for breeding with regard to the mutant mdr1-1Δ allele. Furthermore, it is not necessary to genotype Elos for the mutant mdr1-1 Δ allele before the

administration of drugs which are P-glycoprotein substrates as it is recommended for Collies and other herding dog breeds from the Collie lineage (Neff et al., 2004).

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Table 1. Distribution of the proportions of Old English Sheepdog (OES) genes (%) in the sampled Elos (n_s = 177) and in the current Elo population (n_p = 2973)

Proportion of OES genes (%)	Dam A		Sire B		Sire C		Dam D		OES Founders	
	n_s	n_p	n_s	n_p	n_s	n_p	n_s	n_p	n_s	n_p
0	-	31	26	377	173	2876	11	278	-	3
0 - 5	4	67	151	2596	1	8	166	2618	2	62
5 - 10	22	199	-	-	3	54	-	77	15	170
10 - 15	44	635	-	-	-	9	-	-	25	306
15 - 20	60	834	-	-	-	-	-	-	51	655
20 - 25	46	1116	-	-	-	18	-	-	52	831
25 - 30	1	80	-	-	-	-	-	-	31	786
30 - 40	-	11	-	-	-	-	-	-	1	134
40 - 50	-	-	-	-	-	8	-	-	-	18
50 - 60	-	-	-	-	-	-	-	-	-	8
Mean (%)	16.31	17.42	1.02	1.26	0.12	0.45	1.31	1.66	18.76	20.79

Dam A = female purebred OES founder

Sire B = male OES founder, sire of the purebred son of Dam A

Sire C = male purebred OES founder

Dam D = female OES crossbred founder

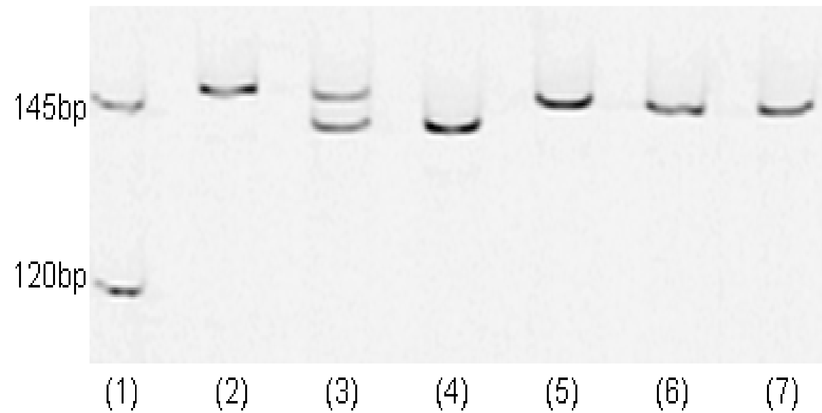

Figure 1. PCR fragments with lengths of 148 bp (wild-type MDR1 allele) and 144 bp (mutant mdr1-1Δ allele) after polyacrylamide gel elektrophoresis.

(1) IRD700-labelled DNA ladder

(2) – (4) Different *MDR1* genotypes from Collies used as reference:

(2) Collie MDR1/MDR1; (3) Collie MDR1/mdr1-1Δ; (4) Collie mdr1-1Δ/mdr1-1Δ

(5) – (7) Analysed Elo blood samples homozygous for the wild-type MDR1 allele with PCR fragment lengths of 148 bp:

(5) Elo No. 9 (gene contributions: 13.68% by Dam A, 1.13% by Sire B, 3.13% by Sire C, 1.67% by Dam D)

(6) Elo No. 46 (gene contributions: 20.02% by Dam A, 2.45% by Sire B, 0.00% by Sire C, 2.74% by Dam D)

(7) Elo No. 48 (gene contributions: 18.76% by Dam A, 2.35% by Sire B, 0.00% by Sire C, 0.79% by Dam D)

Chapter 4

Haplotype analysis of the *MDR1* flanking region in the dog breed Elo

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Haplotype analysis of the *MDR1* flanking region in the dog breed Elo

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Running head Haplotype analysis of the *MDR1* region

Summary

A deletion mutation in the canine multidrug resistance (*MDR1*) gene provokes drug sensitivity in several dog breeds from the Collie lineage. A haplotype of four microsatellites containing this mdr1-1Δ mutation was conserved among affected breeds. In this study, we analysed the haplotypes of the *MDR1* flanking region of 177 dogs of the breed Elo which is composed of several dog breeds including the Old English Sheepdog from the Collie lineage. We detected a haplotype in the Elo breed which had previously been associated with the mutant mdr1-1Δ allele in Old English Sheepdogs. Using a regression analysis for the probability of the haplotype on the proportion of genes of the founder breeds, we could exclude the Old English Sheepdog as origin of this haplotype for the Elo breed. The *MDR1* flanking region could be traced back to the Japanese Spitz as one of the founder dog breeds of the Elo and thus, we ruled out the introgression of the mdr1-1Δ mutation into the dog breed Elo through the Collie lineage.

Keywords: *MDR1*, dog, haplotype, Elo, Old English Sheepdog.

Zusammenfassung

Eine Deletionsmutation im *MDR1*(multidrug resistance)-Gen des Hundes verursacht Arzneimittelüberempfindlichkeit bei Collies und deren verwandten Rassen. Diese mdr1-1Δ-Mutation befindet sich in einem für die Collie-Rassen konservierten Genomabschnitt. In dieser Studie wurden die Haplotypen der *MDR1*-Region bei 177 Hunden der Rasse Elo untersucht. Da der Elo aus mehreren Hunderassen, einschließlich des Old English Sheepdog

aus der Collie-Linie, gezüchtet wurde, kann überprüft werden, inwieweit sich dieser bei Collie-Rassen konservierte Haplotyp in einer Kreuzungspopulation erhält und wie eindeutig diese Haplotypen für die Collie-Linien sind. Wir fanden einen Haplotyp bei den untersuchten Elos, der mit dem mutierten mdr1-1Δ-Allel bei Old English Sheepdogs assoziiert ist. Mittels einer Regressionsanalyse, bei der der Zusammenhang zwischen der Wahrscheinlichkeit dieses Haplotyps und der Genanteile der Gründerrassen ermittelt wurde, konnten wir den Old English Sheepdog als Ursprung für diesen Haplotyp für die Rasse Elo ausschließen. Die *MDR1* umgebende Region konnte auf den Japanspitz als eine der Gründerrassen des Elos zurückgeführt werden. Aus diesem Grund konnten wir ausschließen, dass der die mdr1-1Δ-Mutation umgebende Genombereich über Einkreuzung des Old English Sheepdogs in die Hunderasse Elo eingeführt wurde. Somit kann die mdr1-1Δ-Mutation bei Elos nicht vorkommen.

Schlüsselwörter *MDR1*, Hund, Haplotyp, Elo, Old English Sheepdog.

Introduction

The canine multidrug resistance gene, *MDR1* (*ABCB1*, ATP-binding cassette sub-family B member 1), is located on *Canis familiaris* chromosome 14 and composed of 28 exons. It encodes the transmembrane protein pump P-glycoprotein which is an integral component of the blood-brain barrier. A 4-bp deletion mutation in the fourth exon of the *MDR1* gene was found to be the cause of drug sensitivity in dogs (Mealey et al., 2001; Roulet et al., 2003). This mdr1-1Δ mutation causes a frame-shift accompanied by multiple premature stop codons resulting in a severely truncated P-glycoprotein composed of < 10% of the wild-type amino acid sequence and loss of its protecting function in the blood-brain-barrier. P-glycoprotein is an ATP-dependent drug transporter that extrudes a variety of substrates from brain tissue back into the capillary lumen resulting in lower concentrations of these substrates in the brain. If the protecting function of P-glycoprotein gets lost, P-glycoprotein substrates can accumulate in the brain tissue and provoke neurotoxic adverse effects (Cordon-Cardo et al., 1989; Schinkel, 1998; Fromm, 2000, 2004). More than 50 therapeutic drugs are known substrates of P-glycoprotein. Some of these drugs like ivermectin (Mealey et al., 2001), vincristine, vinblastine, doxorubicin (Mealey et al., 2003), doramectin (Yas-Natan et al., 2003), loperamide (Sartor et al., 2004), digoxin (Henik et al., 2006), moxidectin (Geyer et al., 2005a)

and dexamethasone (Mealey et al., 2007) are described to cause neurotoxic reactions after routine doses in dogs with the deletion mutation in the *MDR1* gene. Several British herding dog breeds from the Collie lineage such as rough-coated and smooth-coated Collie, Shetland Sheepdog, Old English Sheepdog or Australian Shepherd and other related breeds are affected by the mdr1-1Δ mutation (Neff et al., 2004; Geyer et al., 2005b; Kawabata et al., 2005; Mealey et al., 2005). The Old English Sheepdog breed showed low mdr1-1Δ allele frequencies of 3.6% (Neff et al., 2004) and 6.3% (Geyer et al., 2005b), respectively, but no homozygous mdr1-1Δ/mdr1-1Δ genotype. Neff et al. (2004) demonstrated allelic association between the mdr1-1Δ allele and a haplotype containing the four closest microsatellites (*C14.866*, *REN103E18*, *REN144I15*, *G01506*) to *MDR1*. The conservation of allelic association indicated that the mdr1-1Δ allele arose once and that the affected breeds shared the allele identical by descent. The mutation probably originated in a working sheepdog in Great Britain before the beginning of pure breeding in 1873. From there, the mdr1-1Δ allele was dispersed over the dog breeds from the Collie lineage which shared the same sheepdog ancestors. Old English Sheepdogs with the mdr1-1Δ mutation developed three breed-specific haplotypes. Together with the fact, that the Old English sheepdogs showed a diversity of haplotypes (4 haplotypes from 10 mutant chromosomes), this breed was assumed to segregate the mdr1-1Δ allele for many generations and to be one of the first breeds which diverged from the Collie lineage (Neff et al., 2004). The Elo is a newly developed German dog breed based on 16 founder animals stemming from nine different dog breeds as following: Eurasian Dog, Old English Sheepdog, Chow-Chow, Samoyed, Dalmatian, Pekingese, Pomeranian Dog, German Spitz and Japanese Spitz. Four dogs with Old English Sheepdog ancestry participated in the foundation of the Elo (Kaufhold et al., 2005). Because of the ancestry from the Old English Sheepdog breed, the Elo was suspected to carry the mutant mdr1-1Δ allele.

The purpose of this study was to determine whether haplotypes of the Old English Sheepdog, which were found to be associated with the mutant mdr1-1Δ allele, could be recovered in the Elo breed. These haplotypes and the inbreeding on founder animals in the Elo breed imply risks for the Elos being susceptible for the mdr1-1Δ mutation. So the haplotype analysis was used as a tool to test for the presence of the Collie lineage haplotype for the *MDR1* region as supposed by Neff et al. (2004).

Materials and Methods

Genomic DNA that was extracted from EDTA-blood samples of 177 clinically healthy, client-owned Elos using the QIAamp® 96 Spin Blood Kit (Qiagen, Hilden, Germany) was used for haplotype analysis. Available pedigree data included the sampled Elos as well as the Elo population from the beginning of breeding in 1987 until the end of the year 2005. Gene contributions by the Old English Sheepdog breed in the sampled Elos were representative for the current Elo population. All dogs of the sample were analysed for the mutant mdr1-1Δ allele in a previous study (Fecht et al., 2007). Four microsatellite markers were used for haplotyping as previously published (Neff et al. 2004). The positions of the four markers and *MDR1* were determined in the sequence of CFA14 obtained from the NCBI genome database (GenBank accession no. NC_006596). The order of loci was according to the dog genome assembly 2.1 and the haplotype had to be re-ordered as follows: *C14.866-REN144I15-MDR1-REN103E18-G01506* with positions on CFA14 at 15.6, 16.5, 16.6, 17.7 and 19.3 Mb (dog genome assembly 2.1). Primer sequences of the microsatellite markers used for amplification are according to dog genome assembly 2.1. For the analysis of the marker alleles, the PCR products were size-fractionated by gel electrophoresis on an automated sequencer (LI-COR, Lincoln, NE, USA) using 6% polyacrylamide denaturing gels (RotiphoreseGel40, Carl Roth, Karlsruhe). The marker alleles were assigned by visual examination. The analysis of the pedigree data was carried out using the programme OPTI-MATE, version 3.87 (Wrede and Schmidt, 2003) to calculate gene contributions by different founder dog breeds, inbreeding coefficients, indices of pedigree completeness and the contributions to the inbreeding coefficients by the Old English Sheepdog and all other founder breeds for selected individuals (Table 1) as well as for the current Elo population defined by the dogs born from 1994 until 2005. Allele frequencies, their confidence intervals, deviations from Hardy-Weinberg equilibrium and linkage disequilibrium among loci were assessed using the ALLELE procedure of SAS/Genetics, version 9.1.3 (Statistical Analysis System, SAS Institute, Cary, NC, USA). Haplotypes were inferred using the HAPLOTYPE procedure of SAS/Genetics, version 9.1.3. Statistical evaluation of the gene contributions by the different founder dog breeds and their association with the haplotype “247-255-148-173-159” was made by stepwise forward/backward regression analysis employing GLM (general linear model) of SAS, version 9.1.3. The probability to observe the haplotype “247-255-148-173-159” in a

specific individual dog was used as dependent variable and tested for the influence of gene contributions by the different founder breeds. First the influence of each founder breed was calculated separately. Finally, the breeds significant in simple analysis of variance were analysed simultaneously. The final model explaining the origin of the haplotype "247-255-148-173-159" best included the gene contributions of Pomeranian Dog, Pekingese and Japanese Spitz.

$Y_{ijkl} = \mu + b_1POM_i + b_2PEK_j + b_3JSP_k + e_{ijkl}$

Y_{ijkl}	probability of the haplotype "247-255-148-173-159" for the ijkl-th dog in the 177 randomly sampled Elos
μ	model constant
b_1 to b_3	linear regression coefficients
POM_i	gene contribution by Pomeranian Dog
PEK_j	gene contribution by Pekingese
JSP_k	gene contribution by Japanese Spitz
e_{ijkl}	random residual effects

Results and Discussion

The analysis revealed 35 different haplotypes in 177 Elos and 13 haplotypes with a frequency > 1% (Table 2). The "247-251-148-178-159" haplotype was the most frequent for the Elo breed with 31.87 %. The haplotype "247-255-148-173-159" that had been associated with the mutant mdr1-1Δ allele in the Old English Sheepdog breed (Neff et al., 2004) was found in Elos with a frequency of 2.26 %. However, none of the Elos was homozygous for this haplotype. Other haplotypes that had previously been associated with the mutant mdr1-1Δ allele could not be found in the present study. To evaluate the origin of the haplotype "247-255-148-173-159", we analysed the gene contributions and the inbreeding by founder dog breeds. The eight Elos with the haplotype "247-255-148-173-159" had low gene contributions by the Old English Sheepdog breed ranging between 8.32 % and 16.82 % compared to the mean value of gene contribution by the Old English Sheepdog breed in the current Elo population of 20.81%. Otherwise, each Elo with this haplotype had noticeable high gene contributions by Japanese Spitz, Pomeranian Dog and Pekingese (Table 1). The inbreeding coefficient for the current Elo population was 13.3 % with a pedigree completeness of 28.4 %

for eleven generations. The Old English Sheepdog breed contributed 8.23 % to the inbreeding coefficient of the current Elo population, whereas the Japanese Spitz contributed only 2.01 %. The eight Elos with the haplotype “247-255-148-173-159” had inbreeding coefficients between 5.9 % and 17.5 % with a mean of 11 % (Table 1). The contributions to the inbreeding coefficients of these eight Elos by the Old English Sheepdog breed ranged from 0.83 % to 10.57 %. The Japanese Spitz contributed above average of the reference population to the inbreeding coefficients of six of the eight Elos with values ranging from 11.16 % to 27.95 %. For three of the eight Elos, the Japanese Spitz had the largest contribution to the inbreeding coefficients. It was conspicuous that none of the eight dogs was homozygous for the haplotype “247-255-148-173-159” despite of a high inbreeding coefficient. In addition, a high contribution to inbreeding by Japanese Spitz may let expect highly conserved genomic regions and therefore homozygosity of this haplotype. The subsequent regression analysis evaluated the influence of the gene contributions by the different founder breeds on the probability to observe the haplotype “247-255-148-173-159”. After the exclusion of six from nine founder breeds due to very low and insignificant contributions to the variance explained, the final model included the gene contributions by Pomeranian Dog, Pekingese and Japanese Spitz and was significant with a p-value < 0.0001. The regression coefficient of the haplotype probability on the gene contribution by the Japanese Spitz was significant (p = 0.0314). The other two dog breeds contributed to the total variance of the model (11.8 %) but these gene contributions were not significant (p = 0.11). Therefore, the analysis resulted in the conclusion that the haplotype “247-255-148-173-159” traced back to the Japanese Spitz as one of the founder dog breeds of the Elo. In addition, we showed in a previous analysis that these 177 dogs of the breed Elo did not carry the mdr1-1Δ mutation (Fecht et al. 2007). Contrary to the assumption that the haplotype “247-255-148-173-159” could originate in the Old English Sheepdog breed because the *MDR1* flanking region was expected to be highly conserved and this haplotype was supposed to be associated with the mdr1-1Δ mutation in Old English Sheepdogs (Neff et al. 2004), we could verify the existence of the haplotype “247-255-148-173-159” in Elos without the mdr1-1Δ mutation. So we suggest that this genomic region is not inevitably associated with the mutant mdr1-1Δ allele. Since we detected this haplotype in the dog breed Elo and could assume that it originated in the Japanese Spitz, it might be likely that other dog breeds also have the haplotype “247-255-148-

173-159” without being affected by the mdr1-1Δ mutation. For a general statement about the association of the mutant mdr1-1Δ allele with the reported haplotypes, especially the haplotype “247-255-148-173-159”, further analyses of the *MDR1* flanking region are necessary in dog breeds affected by the mdr1-1Δ mutation as well as in non-affected dog breeds.

Thus, the existence of the mdr1-1Δ mutation in the Elo population could be excluded for the following reasons. No mdr1-1Δ allele had been detected in the sample of 177 Elos in a previous study (Fecht et al., 2007). The same *MDR1* flanking region which had previously been found in Old English Sheepdogs with the mdr1-1Δ mutation matched with a Japanese Spitz haplotype without the mdr1-1Δ mutation in this study. This breed has not been pulled together with the mdr1-1Δ mutation associated with drug sensitivity up to the present.

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Table 1: Gene contributions by eight founder dog breeds, inbreeding coefficients (F), indices of pedigree completeness (P) and the contribution to the inbreeding coefficients by the Old English Sheepdog (F_{OES}) and the Japanese Spitz (F_{JSP}) for the eight Elos with the haplotype "247-255-148-173-159" (in %). (OES = Old English Sheepdog, EUR = Eurasian Dog, CHO = Chow-Chow, POM = Pomeranian Dog, GSP = German Spitz, DAL = Dalmatian, JSP = Japanese Spitz, PEK = Pekingese)

Animal (No.)	OES	EUR	CHO	POM	GSP	DAL	JSP	PEK	F	P	F_{OES}	F_{JSP}
1	8.32	25.72	3.09	16.99	0.79	0.00	28.10	16.99	17.5	43.8	0.83	13.46
2	8.42	25.96	3.14	18.74	0.00	0.00	25.00	18.74	11.2	28.6	2.09	27.95
3	9.30	26.63	3.14	14.84	0.00	6.25	25.00	14.84	14.0	33.4	1.52	11.16
4	9.30	26.63	3.14	14.84	0.00	6.25	25.00	14.84	14.0	33.4	1.52	11.16
5	10.18	31.20	3.53	14.65	0.79	0.00	25.00	14.65	12.0	39.2	2.37	26.12
6	14.34	39.53	4.31	11.14	0.79	0.00	18.75	11.14	7.5	48.1	6.76	21.30
7	16.82	45.37	6.16	9.18	0.79	0.00	12.50	9.18	5.9	38.8	10.57	0
8	16.82	45.37	6.16	9.18	0.79	0.00	12.50	9.18	5.9	38.8	10.57	0
Mean	11.69	33.30	4.08	13.70	0.49	1.56	21.48	13.70	11.0	38.0	4.53	12.50

Table 2: Frequencies of haplotypes inferred for the Elo breed.

No.	Haplotype	Frequency (%)	Standard error	95 % Confidence limits	
1	**247-251-148-178-159**	**31.87**	0.025	0.270	0.367
2	**247-251-148-173-159**	**14.63**	0.019	0.109	0.183
3	**241-251-148-180-165**	**12.14**	0.017	0.087	0.155
4	251-251-148-178-157	7.96	0.014	0.051	0.108
5	247-259-148-180-155	4.80	0.011	0.026	0.070
6	243-255-148-177-161	4.52	0.011	0.024	0.067
7	247-257-148-173-159	3.67	0.010	0.017	0.056
8	247-259-148-173-151	3.40	0.010	0.015	0.053
9	239-257-148-173-159	2.26	0.008	0.007	0.038
10	**247-255-148-173-159**	**2.26**	0.008	0.007	0.038
11	247-255-148-177-161	1.42	0.006	0.002	0.027
12	247-251-148-173-155	1.41	0.006	0.002	0.026
13	241-259-148-173-151	1.40	0.006	0.002	0.026

The loci order is *C14.866-REN144I15-MDR1-REN103E18-G01506*. Haplotypes 1, 2 and 3 are in bold as they are the most frequent haplotypes for the Elo breed. Haplotype 10 is in bold as it is the only haplotype which was previously associated with the mdr1-1Δ allele. Only haplotypes with a frequency >1% are given.

Chapter 5

Searching for functional polymorphisms in the exons 12, 21 and 26 of the canine *MDR1* gene

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Searching for functional polymorphisms in the exons 12, 21 and 26 of the canine *MDR1* gene

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Abstract

Numerous single nucleotide polymorphisms (SNPs) have been identified in the human multidrug resistance (*MDR1*, *ABCB1*) gene. Three of these SNPs [c.1236C>T, c.2677G>T/A and c.3435C>T] were found to be in linkage disequilibrium and the synonymous substitution c.3435C>T was associated with decreased *MDR1* function and reduced mRNA and protein expression in some tissues. Besides the mdr1-1Δ mutation associated with multiple drug sensitivity in several dog breeds, no further sequence variations in the canine *MDR1* gene have been described yet. To analyse whether functional polymorphisms exist in the exons 12, 21 and 26 of the canine *MDR1* gene analogically to the findings in the orthologous human gene, sequence analysis was performed in two Collies and six dogs of the Elo breed. We detected a nonsynonymous A to G exchange in exon 26 (c.3508A>G) which causes an amino acid substitution from methionine to valine at position 1147. Subsequently, we analysed 88 Elo dogs and 65 dogs of different breeds for this A to G transition using restriction fragment length polymorphism (RFLP). The presence of the G-allele in the analysed Elos with a frequency of 57.95% was mainly influenced by the founder breed Samoyed. Furthermore, the mutant allele was found in Labrador Retrievers, Do-Khyis, Dalmatians, German Wirehaired Pointers, Hovawarts and Border Collies. The G-allele could not be detected in Collies, German Shepherds, Dachshunds, Tibetan Terriers, English Cocker Spaniels, Irish Wolfhounds, Jack Russell Terriers, Boxers and Kromfohrlanders. In the analysis of protein alignments used for the prediction of possible impact of the amino acid substitution on the structure and function of the protein this sequence variant was predicted to be benign. Quantitative RT-PCR revealed no significant difference between the relative expression levels

of *MDR1* in liver biopsies of two Elos with the A/A genotype and two Elos with the G/G genotype. Nevertheless, effects of the newly detected c.3508A>G SNP on expression, structure or functionality of *MDR1* mRNA and/or protein and the presence of further functional polymorphisms in the canine *MDR1* gene could not be completely excluded in this study.

Introduction

The superfamily of ABC (ATP-binding cassette) transporters have been of particular interest for research work in humans and animals, i.e. mice and dogs, for several years now. The *MDR1* gene (*multidrug resistance* gene, also known as *ABCB1* gene, *ATP-binding cassette sub-family B member 1*) and its gene product P-glycoprotein are the most thoroughly analysed among the ABC transporters (Pauli-Magnus and Kroetz, 2004).

The physiological role of P-glycoprotein is the protection of the organism from toxic xenobiotics. P-glycoprotein is normally expressed in various mammalian tissues including brain capillary endothelial cells (Cordon-Cardo et al., 1989), the apical border of intestinal epithelial cells (Li et al., 1999), biliary canalicular cells (Thiebaut et al., 1987), renal proximal tubular epithelial cells (Hori et al., 1993), placenta (Lankas et al., 1998), and testes (Melaine et al., 2002). P-glycoprotein confers protection by limiting the uptake of compounds from the gastrointestinal tract and by contributing to their excretion via the liver, kidneys, and intestine. Moreover, P-glycoprotein in the blood-brain-barrier and other blood-tissue barriers protects sensitive organs from exposure to toxic compounds that may have entered the bloodstream (Schinkel, 1997). P-glycoprotein actively extrudes selected xenobiotics from within the cell back into the lumen of brain capillary, intestine, bile canaliculus, or renal tubule.

More than 50 structurally different therapeutic drugs are known substrates for human and murine P-glycoprotein. Because of the high degree of homology of P-glycoprotein between species, the same drugs are expected to be substrates of the canine P-glycoprotein (Mealey et al., 2007). Degree of expression and the functionality of the *MDR1* gene product can directly affect the therapeutic effectiveness of such agents because they play an important role for the physiological cell protection during drug therapy (Hoffmeyer et al., 2000).

The canine *MDR1* gene attracted interest for research work after several descriptions of ivermectin neurotoxicity in Collies and the observation that affected dogs had elevated

concentrations of ivermectin in the central nervous system indicating that ivermectin neurotoxicity was caused by a defect in the blood-brain barrier (Preston, 1983; Seward, 1983; Pulliam et al., 1985).

The canine *MDR1* gene is located on CFA (*Canis familiaris* autosome) 14 and composed of 28 exons. The human *MDR1* (*ABCB1*) gene is located on HSA (*Homo sapiens* autosome) 7. The product of the human *MDR1* gene is among all species documented in data banks the most similar to that of the canine gene. Human and dog P-glycoprotein display 91 % overall homology, with non-consensus residues being located outside the functional segments and are composed each of 12 transmembrane domains and two nucleotide binding domains (Roulet et al., 2003). A 4-bp deletion mutation in the fourth exon of the *MDR1* gene was found to be the cause of ivermectin sensitivity in dogs (Mealey et al., 2001; Roulet et al., 2003). This mdr1-1Δ mutation causes a frame-shift accompanied by multiple premature stop codons resulting in a severely truncated P-glycoprotein composed of < 10% of the wild-type amino acid sequence. The remainder of the protein lost its protecting function, e.g. in the blood-brain-barrier. Neurotoxic side effects are provoked in dogs with the mdr1-1Δ mutation in the case of drug therapy with P-glycoprotein substrates because of accumulation of these substrates in brain tissue. Although both research groups screened the whole cDNA of the canine *MDR1* gene, there was no evidence on further sequence variations in this gene in the analysed Collies.

In humans, many studies were concerned with sequence variations in the *MDR1* gene. DNA sequence variations cause phenotypic changes by multiple mechanisms, e.g. by changing the encoded protein sequence, or by affecting gene regulation, mRNA processing, and translation (Wang and Sadée, 2006). Many of the detected polymorphisms in humans do not show effects on expression or function of *MDR1* but some implicate modified protein levels or functionality, e.g. the SNP in exon 26 (c.3435C>T) (Hoffmeyer et al., 2000). This research group performed the first systematic screening of the *MDR1* gene for the presence of polymorphisms by sequencing all 28 exons including the core promoter region and flanking intron-exon boundaries. Of the 15 identified SNPs, the research group identified two SNPs at wobble positions with no amino acid changes [exon 12 (c.1236C>T) and exon 26 (c.3435C>T)] and found an association of the SNP c.3435C>T with a modified level of intestinal *MDR1*-expression. Although results are not always consistent, most studies suggest

that the c.3435C>T transition is associated with decreased *MDR1* function and reduced mRNA and/or protein expression in some tissues (Pauli-Magnus and Kroetz, 2004). Wang and Sadée (2006) reported that the two synonymous SNPs [exon 12 (c.1236C>T) and exon 26 (c.3435C>T)] are in linkage disequlibrium with a nonsynonymous SNP in exon 21 (c.2677G>T/A) which causes an amino acid change (899Ala>Ser/Thr). Furthermore, they stated that c.3435C>T is a functional SNP that decreases mRNA stability, thereby decreasing *MDR1* mRNA and/or protein levels, by analysis of allele-specific expression in liver autopsy samples and in vitro expression experiments.

It is not yet known whether polymorphisms in the canine *MDR1* gene exist like in the human gene. In addition to the known deletion mutation in the fourth exon, further sequence variations in the canine *MDR1* gene might also be found which modify the structure or expression levels of the *MDR1* mRNA and/or protein and thereby change the function of P-glycoprotein. Therefore, the purpose of this study was to search for functional polymorphisms by sequencing three exons of the canine *MDR1* gene which were in accordance to the orthologous human DNA sequences including the three linked SNPs.

Materials and Methods

Animals

Blood samples of 88 clinically healthy, client-owned Elo dogs were used for this analysis. For all Elos, the existence of the mutant mdr1-1Δ allele was excluded in a previous study (Fecht et al., 2007). Additionally, blood samples of eleven Collies, two Border Collies, four German Shepherds, four Do-Khyis, four German Wirehaired Pointers, four Dachshunds, four Labrador Retrievers, four Tibetan Terriers, four English Cocker Spaniels, four Irish Wolfhounds, four Dalmatians, four Jack Russell Terriers, four Boxers, four Hovawarts and four Kromfohrlanders were available for genotyping of exon 26. Six of the eleven Collies were homozygous for the mutant mdr1-1Δ allele and three Collies showed the heterozygous genotype. Two Collies, the four German Shepherds and one of the Border Collies were homozygous for the wild-type MDR1 allele. The remaining dogs were not tested for the presence of the mdr1-1Δ allele. Genomic DNA was extracted from EDTA (ethylenediaminetetraacetic)-anticoagulated blood using the NucleoSpin Kit 96 Blood Quick Pure Kit (Macherey-Nagel, Düren, Germany). For

the analysis of *MDR1* expression, four liver biopsies of previously genotyped Elos dogs were available.

Sequence analysis

We chose the canine exonic sequences according to the dog genome assembly 2.1 which corresponded to the orthologous human exons of *MDR1* including the SNPs c.1236C>T, c.2677G>T/A and c.3435C>T to search for polymorphisms in dogs by sequencing genomic DNA of two Collies and six Elos. Therefore, we aligned the canine *MDR1* genomic DNA reference sequence (GenBank accession no. NC_006596.2) with the canine *MDR1* reference mRNA (GenBank accession no. NM_001003215.1) and the human *MDR1* (*ABCB1*) reference mRNA (GenBank accession no. NM_000927.3) obtained from the NCBI database. For the exact localization of the exon/intron boundaries the mRNA-to-genomic alignment program Spidey (http://www.ncbi.nlm.nih.gov/IEB/Research/Ostell/Spidey/index.html) was used. The human sequences which included the three linked SNPs [c.1236C>T, c.2677G>T/A and c.3435C>T] matched with the sequences of the exons 12, 21 and 26 of the canine *MDR1* gene. Three primer pairs encompassing the exons 12, 21 and 26 including the flanking intronic regions were designed using the PRIMER3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) and the Repeatmasker 3.1.0 (http://www.repeatmasker.org/cgi-bin/WEBRepeatMasker) for the detection and masking of repetitive elements (Table 1). The PCR reactions were performed in a total volume of 30 µl using 2 µl (~ 20 ng/µl) genomic DNA, 3 µl 10x PCR buffer, 0.72 µl DMSO, 0.6 µl (10 µM) of each primer, 0.6 µl dNTPs (10mM each) and 0.2 µl (5 U/µl) *Taq* Polymerase (Qbiogene, Heidelberg, Germany). The reactions were performed in MJ Research thermocyclers (MJ Research, Watertown, MA, USA) and started with 5 min initial denaturation at 95°C, followed by 36 cycles at 95 °C for 30 sec, annealing temperature (Ta) at 60°C for 1 min, and extension at 72 °C for 45 sec. The PCR was completed with a final cooling at 4°C for 10 min. The PCR products were cleaned using a Sephadex G50 filtration (GE Healthcare, Freiburg, Germany) and directly sequenced with the DYEnamic ET Terminator Cycle Sequencing kit (GE Healthcare) on a MegaBACE 1000 capillary sequencer (GE Healthcare). Sequence data was analyzed using the Sequencher 4.7 program (GeneCodes, Ann Arbor, MI, USA).

RFLP (restriction fragment length polymorphism)

The single nucleotide polymorphism (SNP) in exon 26 modified the recognition site of the restriction enzyme *Bsg* I (**G**TGCAG$(N)_{16}$). The modification allowed the analysis of the SNP using restriction fragment length polymorphism (RFLP) in 88 Elos and 65 dogs of different breeds. The restriction enzyme *Bsg* I cut the PCR product if a G exists at the substitution position. Digestion of the PCR product with the restriction enzyme *Bsg* I and size-fractionation by gel electrophoresis allowed distinguishing the two different alleles.

10 µl of the PCR product was digested using *Bsg* I (New England Biolabs, Frankfurt/Main, Germany) and separated on a 2% agarose gel so that a 372-bp fragment for the A/A genotype, 258-bp and 116-bp fragments for the G/G genotype, and 372-bp, 258-bp and 116-bp fragments for the G/A genotype were observed (Figure 1).

Quantitative Reverse Transcriptase (qRT)-PCR analysis of MDR1 expression

For the analysis of *MDR1* expression, liver biopsies of four Elos, each two with the A/A and the G/G genotype, were available. RNA of liver biopsies was isolated directly with the RNeasy 96 Universal Tissuekit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The reverse transcription into cDNA was performed by using 200 U SuperScript III Reverse Transcriptase (Invitrogen, Karlsruhe, Germany), an oligo-dT primer, and 10 µl of the isolated RNA in a 20-µl reaction. A single PCR assay was used for quantification of the *MDR1* gene transcript (GenBank NM_001003215.1) using a forward primer situated in exon 12 (5'-GAC CGT GCA GCT GAT GCA-3') and a reverse primer in exon 13 (5'-GGT CCT AAT GTC CTG TCC ATC AA-3') amplifying an 79-bp product with a VIC-labelled TaqMan minor groove binding (MGB) probe (Applied Biosystems, Darmstadt, Germany) located at the boundary of exon 12 and 13 (5'-ACA GAT GGC ATG GTC T-3'). Canine *GAPDH* transcript was determined as endogenous control using a forward primer situated in exon 4 (5'-GGC ACA GTC AAG GCT GAG AAC-3') and a reverse primer in exon 5 (5'-CCA GCA TCA CCC CAT TTG AT-3') amplifying a 101-bp product in combination with a FAM-labelled TaqMan MGB probe (Applied Biosystems) spanning the boundary of exon 4 and 5 (5'-TCC AGG AGC GAG ATC-3') according to the canine reference mRNA sequence (GenBank NM_001003142). The quantitative reverse transcriptase (qRT)-PCR was carried

out with an ABI 7300 sequence detection system (Applied Biosystems) in a 20-µl reaction containing SensiMix DNA Kit (Quantance Ltd, London, Great Britain), 50 µM forward primer, 50 µM reverse primer, and 10 µM TaqMan probe using an annealing and elongation temperature of 58 °C. The *MDR1* transcript–specific expression was normalised by the canine *GAPDH* expression level (ΔC_T), and the relative expression level was calculated by the $2^{-\Delta\Delta C_T}$ method using the homozygous A/A sample as calibrator (Livak and Schmittgen, 2001). All assays were performed twice in duplicates.

Protein sequence alignments

The program PolyPhen (http://genetics.bwh.harvard.edu/pph/) was used for the predicition of possible impact of the amino acid substitution on the structure and function of the protein via analysis of multiple sequence alignments and protein 3D-structures.

The amino acid sequence of the canine *MDR1* product (GenBank accession no. NP_001003215.1) obtained from the NCBI database (http://www.ncbi.nlm.nih.gov/sites/entrez) with the variants methionine and valine at position 1147 was supplied to PolyPhen for analysis.

Additionally to PolyPhen prediction, we compared the amino acid sequences of dog (GenBank accession no. NP_001003215.1), human (GenBank accession no. NP_000918.2), mouse (GenBank accession no. NP_035205.1), rat (GenBank accession no. NP_036755.2) and sheep (GenBank accession no. NP_001009790.1) using the ClustalW (1.83) multiple sequence alignment from the EMBL toolbox (http://www.ebi.ac.uk/clustalw/index.html) (Figure 2).

Statistical Analyses

For evaluation of the c.3508A>G SNP in exon 26 genotype frequencies, allele frequencies, their confidence intervals, deviations from Hardy-Weinberg equilibrium and linkage disequilibrium among loci were assessed using the ALLELE procedure of SAS/Genetics, version 9.1.3 (Statistical Analysis System, SAS Institute, Cary, NC, USA). Statistical analysis of the gene contributions by the different founder dog breeds and their association with the distribution of the alleles A and G was made by stepwise forward/backward regression analysis employing GLM (general linear model) of SAS, version 9.1.3. The presence of the

allele A or the allele G in a specific individual dog was used as dependent variable and tested for the influence of gene contributions by the different founder breeds. Therefore, the allele A was encoded as 1 and the allele G as 2. First the influence of each founder breed was calculated separately. Finally, the breeds significant in simple analysis of variance were analysed simultaneously. The final model included the gene contributions by Samoyed and Dalmatian.

$Y_{ijk} = \mu + b_1 SAM_i + b_2 DAL_j + e_{ijk}$

Y_{ijk}	presence of allele A or G in the ijk-th dog in the 88 randomly sampled Elos
μ	model constant
b_1, b_2	linear regression coefficients
SAM_i	gene contribution by Samoyed
DAL_j	gene contribution by Dalmatian
e_{ijk}	random residual effects

Statistical evaluation of *MDR1* expression results was performed using the t-test with GLM (general linear model) of SAS, version 9.1.3.

$Y_{ijk} = \mu + Genotype_i + Rep_j + e_{ijk}$

Y_{ijk}	relative expression level of *MDR1*
μ	model constant
$Genotype_i$	fixed effect of genotype (A/A, G/G)
Rep_j	fixed effect of replicate
e_{ijk}	random residual effects

Results

Sequence analysis of the exons 12, 21, 26 was performed in two Collies and six Elos. The analysed DNA sequences of the exons 12 and 21 perfectly matched with the reference sequence of the canine *MDR1* gene (GenBank accession no. NC_006596.2). We found an A to G transition at position 154 in exon 26 (c.3508A>G) in the DNA sequences of four from six Elos. The position of the identified polymorphism correspond to position 3508 of the canine *MDR1* mRNA (GenBank accession no. NM_001003215.1). Three Elos were homozygous G/G, one Elo was heterozygous A/G and two Elos as well as the two Collies

were homozygous A/A. The reference boxer sequence (dog genome assembly 2.1) showed an A at this position and was therefore defined as wild-type.

Thereupon, an analysis of the c.3508A>G SNP in exon 26 was performed in 88 Elos and 65 dogs of different breeds using the RFLP developed. In Elos, the wild-type A-allele was found with a frequency of 42.05%, whereas the G-allele was prevalent with a frequency of 57.95%. Altogether, 66 from 88 Elos (75.0%) were heterozygous or homozygous for the G-allele (Table 2). In most of the analysed breeds the mutated allele could not be detected. In addition to the Elo breed, the G-allele was detected in Labrador Retrievers, Do-Khyis, Dalmatians, German Wirehaired Pointers, Hovawarts and Border Collies (Table 3).

The subsequent regression analysis evaluated the influence of the gene contributions by the different founder breeds on the presence of the allele A or the allele G. After the exclusion of seven from nine founder breeds due to very low and insignificant contributions to the variance explained, the final model included the gene contributions by Samoyed and Dalmatian and was significant with a p-value of 0.0036. Table 4 shows the regression coefficients and error probabilities for the influence of gene contributions by Samoyed and Dalmatian on the presence of the alleles A and G in the analysed 88 Elo dogs. The regression coefficient for the gene contribution by Samoyed was at +0.0201 which indicated a major influence of the founder breed Samoyed for the G-allele. So, an increase by one percent of the gene contribution by Samoyed raises the frequency of the G-allele by 2.01%. The regression coefficient for the gene contribution by Dalmatian was at –0.0519 which indicated a major influence of the founder breed Dalmatian for the A-allele. Consequently, the frequency of the A-allele rises by 5.19% if the gene contribution by Dalmatian increases by one percent.

The nonsynonymous A to G exchange in exon 26 of the canine *MDR1* gene causes an amino acid substitution from methionine to valine at position 1147 in the amino acid sequence (GenBank accession no. NP_001003215.1). For the analysis of possible impact of the modified amino acid sequence on P-glycoprotein, PolyPhen prediction and protein alignment for five selected species using ClustalW were performed. PolyPhen analysis resulted in the prediction that this variant is benign with a PSIC (Position-Specific Independent Counts) score difference of 0.322. The protein alignment showed that only the dog displayed methionine at position 1147 in the amino acid sequence whereas in human, mouse and rat the

amino acid valine was given at the corresponding position in the reference sequence (Figure 2).

To test whether the nonsynonymous c.3508A>G SNP might influence the *MDR1* expression levels liver biopsies from four previously genotyped Elo dogs were taken and analysed by qRT-PCR. The dog showing the lowest relative *MDR1* expression was arbitrarily set to 1. The mean relative expression levels of *MDR1* in the four analysed Elos by individual and genotype are shown in Figure 3. The t-test revealed no significant difference (p = 0.069) between the relative expression levels of Elos with the A/A genotype (1.186 ± 0.121) or the G/G genotype (1.510 ± 0.084).

Discussion

A sample of 88 dogs of the Elo breed was analysed for a newly detected nonsynonymous single nucleotide polymorphism in exon 26 (c.3508A>G) of the canine *MDR1* gene. The mutant G-allele was prevalent in the Elo breed with a frequency of 57.95% which is remarkable because the A-allele is given in the reference sequence of the canine *MDR1* gene. The regression analysis evaluated the influence of the gene contributions by the different founder breeds of the Elo on the presence of the A-allele or the G-allele in the analysed 88 Elos. The analysis resulted in the conclusion that the presence of the G-allele was mainly influenced by the founder breed Samoyed, whereas the presence of the A-allele in the analysed Elos was largely influenced by the Dalmatian founder dogs. Nevertheless, in the RFLP-PCR the G-allele was also found in the analysed Dalmatians, so this breed was also proved to carry both types of alleles. In addition, the G-allele was detected in the breeds Labrador Retriever, Do-Khyi, German Wirehaired Pointer, Hovawart and Border Collie. Due to the small number of genotyped dogs, the prevalence of the mutant allele in other dog breeds remains unknown and has to be analysed if negative effects of the A to G transition on the function of P-glycoprotein will be proved.

The c.3508A>G SNP in exon 26 causes an amino acid substitution in the *MDR1* protein. Protein alignment with five species and the PolyPhen prediction gave no further evidence that the substitution from methionine to valine has effects on structure or function of the protein. Valine is also found at the corresponding position in the reference amino acid sequence of other mammalian species like human, mouse or rat. Thus, valine in dogs may not have great

impact on the functionality of P-glycoprotein since it is usually found at this position for functional proteins in other species. This location does not seem to be highly conserved because in the PolyPhen alignment output there are several amino acids listed for this position including valine, methionine, isoleucine and glutamic acid. It has to be assumed that a substitution from a nonpolar amino acid to another nonpolar amino acid does not have great impact on the structure of a protein. Nevertheless, modifications in amount or character of bonds in the case of an amino acid substitution can alter the stability of the protein structure.

The c.3508A>G SNP in exon 26 of the canine *MDR1* gene is one base position downstream to the c.3435C>T SNP in the orthologous sequence of the human *MDR1* gene. It is possible that the c.3508A>G transition has effects on mRNA and/or protein as it is the case for the c.3435C>T SNP in the human *MDR1* gene. The c.3435C>T polymorphism is a synonymous substitution, so the amino acid sequence is not affected and there are no obvious structure modifications. Anyhow, association of c.3435C>T with decreased *MDR1* function and reduced mRNA or protein expression in some tissues was described (Pauli-Magnus and Kroetz, 2004). Wang et al. (2005) concluded that modification in *MDR1* mRNA expression resulted from changing of mRNA stability.

The analysis of *MDR1* expression in liver biopsies of four dogs gave no evidence that the c.3508A>G SNP has effects on mRNA expression levels. Natural variation in the individual *MDR1* expression might explain the difference between the mean values of both homozygous genotypes. However, a final conclusion about modification of mRNA expression caused by the newly detected SNP is not possible because the statistical power is restricted due to the limited number of samples. Effects on expression, structure or function of *MDR1* mRNA and/or protein are unlikely but can not be completely excluded in this study with computer simulated analysis and measurement of *MDR1* expression levels in four Elos.

This study was restricted to three exons of the canine *MDR1* gene whose orthologous human exonic sequences included three linked SNPs that were associated with decreased *MDR1* function and reduced mRNA and/or protein expression in some tissues (Pauli-Magnus and Kroetz, 2004). Sequence analyses of the complete cDNA and of the promotor region of the *MDR1* gene have to be performed to discover undetected polymorphisms which might have effects on the functionality of the gene product P-glycoprotein by modifying the expression or structure of *MDR1* mRNA and/or protein. The knowledge about functional polymorphisms in

the canine *MDR1* gene is important because the degree of expression and the functionality of the *MDR1* protein can directly affect the therapeutic effectiveness of drugs.

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Table 1. PCR primers for exons 12, 21 and 26 of the canine *MDR1* gene

Exon	Primer	Sequence (5’ -> 3’)	Ta (°C)	PCR product (bp)
12	MDR1_ex12_F	TCAGTGATTCAACCATGTATTGG	60,0	352
	MDR1_ex12_R	AATGTGAGCTGTGCAAATGG		
21	MDR1_ex21_F	CATCATCCTGAAGAAAATCTAGGC	60,0	393
	MDR1_ex21_R	AACACCGTTCTCCAAGCATAGT		
26	MDR1_ex26_F	TGTCCCATGGTAACCTGACA	60,0	372
	MDR1_ex26_R	AAAGTGTAGGCCAGGGAGGT		

Table 2. Allele and genotype frequencies of the c.3508A>G SNP in exon 26 of the canine *MDR1* gene in dogs of the breed Elo (n=88)

		Frequency (%)	Standard error	95 % Confidence limits	
Allele	A	42.05	0.0424	0.3352	0.5057
	G	57.95	0.0424	0.4943	0.6648
Genotype	A/A	25.00	0.0252	0.0226	0.1229
	A/G	34.09	0.0252	0.0226	0.1229
	G/G	40.91	0.0252	0.0226	0.1229

Table 3. Distribution of genotypes of the c.3508A>G SNP in 65 dogs of different breeds

Dog breed	Number of dogs	Distribution of genotypes		
		A/A	A/G	G/G
Collie	11	11	0	0
German Shepherd	4	4	0	0
Dachshund	4	4	0	0
Tibetan Terrier	4	4	0	0
English Cocker Spaniel	4	4	0	0
Irish Wolfhound	4	4	0	0
Jack Russell Terrier	4	4	0	0
Kromfohrlander	4	4	0	0
Boxer	4	4	0	0
Labrador Retriever	4	3	1	0
Do-Khyi	4	3	1	0
Dalmatian	4	2	2	0
German Wirehaired Pointer	4	1	3	0
Hovawart	4	2	1	1
Border Collie	2	1	0	1

Table 4. Regression coefficients (b), standard errors (SE) and error probabilities (p) for the influence of gene contributions by the founder breeds Samoyed and Dalmatian on the presence of the alleles A and G

	Founder breed	b	SE	p
Simple analysis	SAM	0.0216	0.0106	0.0431
	DAL	-0.0539	0.0193	0.0058
Simultaneous analysis	SAM	0.0201	0.0104	0.0558
	DAL	-0.0519	0.0191	0.0075

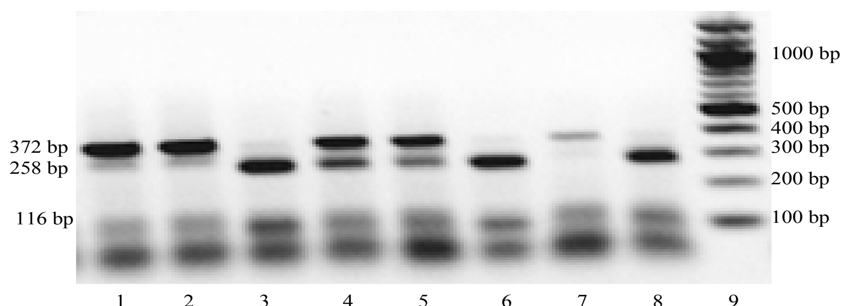

Figure 1. The PCR-RFLP test for the c.3508A>G SNP in exon 26 of the canine *MDR1* gene. The represented Elo dogs show all three genotypes.

1: A/A; 2: A/A; 3: G/G; 4: A/G; 5: A/G; 6: G/G; 7: A/A; 8: G/G; 9: 100bp DNA ladder

```
dog   1135GDNSRVVSHEEIMQAAKEANIHHFIETLPEKYNTRVGDKGTQLSGGQKQRIAIARALVRQ1194
human 1134********Q***VR********A***S**N**S*K*************************1193
mouse 1132*****A******VR********Q**DS**D******************************1191
rat   1131************VR**R*****Q**DS*********************************1190
sheep 1139********Q***EH********S***M**D******************************1198
```

Figure 2. Section of the alignment of the canine *MDR1* protein with known orthologous *MDR1* protein sequences. The amino acid sequences were derived from GenBank entries with the accession nos. NP_001003215.1 (dog), NP_000918.2 (human), NP_035205.1 (mouse), NP_036755.2 (rat) and NP_001009790.1 (sheep). Identical residues are indicated by asterisks.

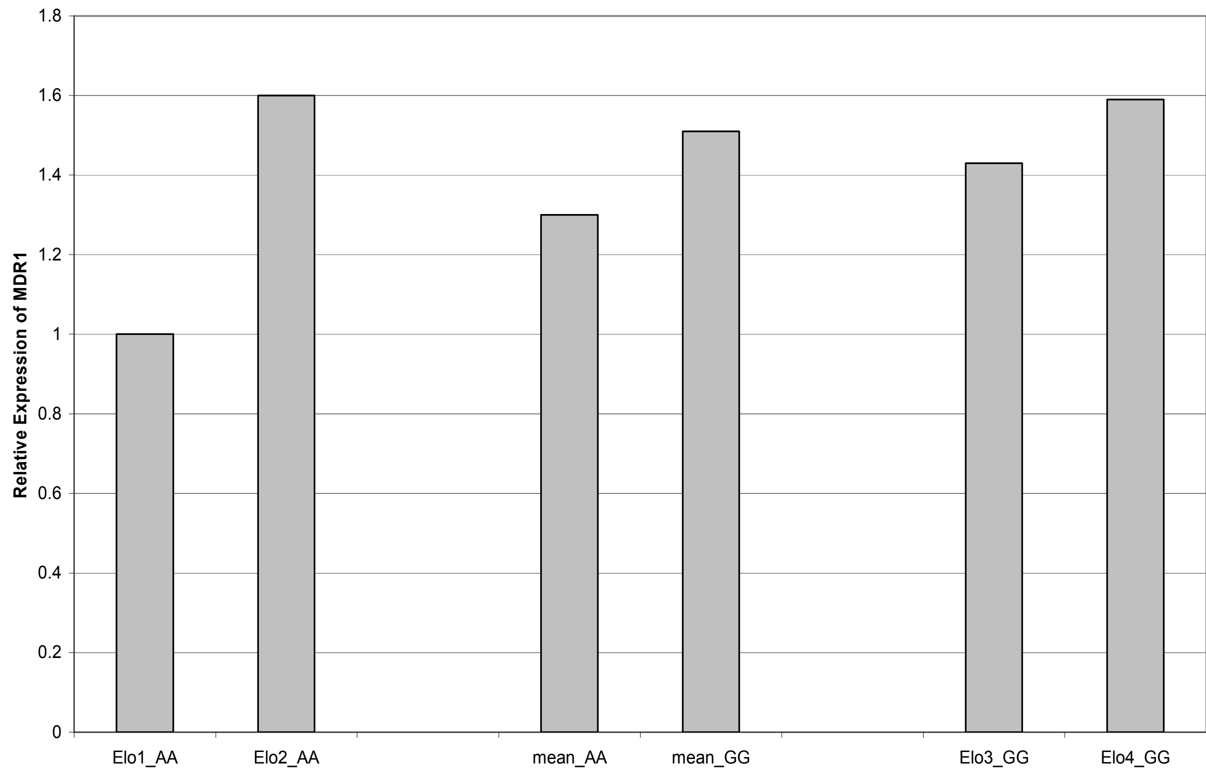

Figure 3. The relative expression of *MDR1* was measured twice by quantitative RT-PCR for each two Elos with the A/A genotype and the G/G genotype. The mean relative expression levels of *MDR1* in the four analysed Elos by individual and genotype are shown. The lowest observed relative *MDR1* expression was arbitrarily set to 1.

Chapter 6

General Discussion

General Discussion

The first aspect of this study was to analyse whether the mdr1-1Δ mutation associated with multiple drug sensitivity caused by a non-functional *MDR1* protein (P-glycoprotein) existed in the newly developed German dog breed Elo. Four dogs with Old English Sheepdog ancestry had participated in the foundation of the Elo (Kaufhold et al., 2005). The Old English Sheepdog contributed with 20.79% to the genes of the current Elo population. The distribution of gene contributions by the four Old English Sheepdog founders in the 177 analysed Elos resembled those in the current Elo population. Hence, a representative sample of the population was obtained. In the last years, several studies were performed concerning the prevalence of the canine mdr1-1Δ mutation in different dog breeds. These studies showed that the mutant mdr1-1Δ allele is presumably restricted to dog breeds of the Collie lineage (Neff et al., 2004; Geyer et al., 2005b; Kawabata et al., 2005; Mealey et al., 2005). Frequencies of the mdr1-1Δ mutation in Collies and related dog breeds from the northwestern United States (Mealey et al., 2002), the entire USA (Neff et al., 2004), France (Hugnet et al., 2004), Germany (Geyer et al., 2005b), Japan (Kawabata et al., 2005) and Australia (Mealey et al., 2005) were determined. Thereby, the mutation in the fourth exon of the canine *MDR1* gene was found in Old English Sheepdogs in low frequencies (Neff et al., 2004; Geyer et al., 2005b). Nevertheless the *MDR1* genotypes of the four founder Old English Sheepdogs were unknown and so an introgression of the mdr1-1Δ mutation into the dog breed Elo through the Old English Sheepdog breed was arguable. Furthermore, the mdr1-1Δ mutation was also detected in other crossing populations based on breeds of the Collie lineage like the Silken Windhound (Neff et al., 2004) and the Wäller (Geyer et al., 2005b). In addition, Neff et al. (2004) found that the mdr1-1Δ allele was identical by descent among affected breeds as evidenced by a single ancestral haplotype. Therefore, the other eight founder breeds of the Elo were not suspected to carry the mutant mdr1-1Δ allele.

In the course of this study, the introgression of the mdr1-1Δ mutation into the dog breed Elo through the Collie lineage could be excluded for the following reasons. Firstly, no mdr1-1Δ allele was observed in the 177 sampled Elos representative for the distribution of Old English Sheepdog genes in the current Elo population. Secondly, the probability that no mdr1-1Δ allele originating in the Old English Sheepdog breed is segregating in the Elo population was

estimated at close to one ($1 - 3.68 \times 10^{-17} \approx 1$). The estimation based on the results of genotyping, the average gene contribution by the Old English Sheepdog breed in the sample and the frequency of the mdr1-1Δ allele in the Old English Sheepdog population. Due to the results of genotyping and estimation of the probability, an undiscovered mdr1-1Δ allele segregating in the Elo population was very unlikely. Thirdly, a haplotype was detected in the sampled Elos which had been associated with the mutant mdr1-1Δ allele in Old English Sheepdogs in a previous study (Neff et al., 2004), but in Elos, this haplotype was found without the mdr1-1Δ mutation. Using a stepwise forward/backward regression analysis for the probability of this haplotype on the proportion of genes of the founder breeds, the Old English Sheepdog could be excluded as origin of this haplotype for the Elo breed. The *MDR1* flanking region was traced back to the Japanese Spitz as one of the founder dog breeds of the Elo.

Neff et al. (2004) assumed a high conservation of the *MDR1* flanking region and listed haplotypes associated with the mutant allele for several dog breeds from the Collie lineage including the Old English Sheepdog. Due to the results of this study, it is arguable if the haplotype detected in Elos is inevitably associated with the mutant mdr1-1Δ allele. Further analyses of the *MDR1* flanking region in affected as well as in non-affected dog breeds are required to make a general statement about the association of the mdr1-1Δ allele with the reported haplotypes.

The second aspect of this study was to search for functional polymorphisms in the canine *MDR1* gene that might have effects on the functionality of P-glycoprotein in addition to the mdr1-1Δ mutation. In humans, numerous single nucleotide polymorphisms (SNPs) in the *ABCB1* (*MDR1*) gene were described which were partly associated with reduced expression levels and function of *MDR1* (Hoffmeyer et al., 2000; Cascorbi et al., 2001; Ito et al., 2001; Kim et al., 2001; Tanabe et al., 2001; Saito et al., 2002; Tang et al., 2002; Kroetz et al., 2003). Additionally, further studies focused on the association of *MDR1* genetic variation with disease, e. g. renal epithelial tumor (Siegsmund et al., 2002), Inflammatory Bowel Disease (Schwab et al., 2003a) or Parkinson´s Disease (Tan et al., 2005). It was hypothesised that genotype-dependent P-glycoprotein expression might contribute to disease susceptibility whereas *MDR1* as marker polymorphism either causes disease or is closely linked to a disease locus (Schwab et al., 2003b). For the canine *MDR1* gene, no genetic variants have been described in the literature yet. Just two research groups analysed the *MDR1* cDNA of Collies

and Beagles only showing the mdr1-1Δ mutation associated with drug sensitivity in the fourth exon of the gene (Mealey et al., 2001; Roulet et al., 2003). For sequence analysis, the canine exonic sequences according to the dog genome assembly 2.1 were chosen which corresponded to the orthologous human exons of *MDR1* including the SNPs c.1236C>T, c.2677G>T/A and c.3435C>T. Many studies were concerned with the effects of these three linked SNPs in humans. Although it provoked no amino acid substitution, and thus it caused no obvious modification in the structure of the protein, the synonymous c.3435C>T transition was found to be the functional SNP associated with reduced function of *MDR1* (Wang and Sadée, 2006). The newly detected c.3508A>G polymorphism in exon 26 of the canine *MDR1* gene is of particular interest because it is one base position downstream to the functional c.3435C>T SNP in the orthologous human exonic sequence and causes an amino acid substitution from methionine to valine. Remarkably, the mutant G-allele was prevalent in the analysed Elo dogs. The presence of the G-allele was mainly influenced by the founder breed Samoyed, whereas the presence of the A-allele in the analysed Elos was largely influenced by the Dalmatian founder dogs. Due to the small number of dogs genotyped, the prevalence of the mutant allele in other dog breeds remains unknown and has to be analysed if negative effects of the G-allele on the function of P-glycoprotein will be found.

There is no doubt that effects of the amino acid substitution on the *MDR1* protein are possible. For a first clarification, computer simulated analysis of the substitution was performed. The program PolyPhen (http://genetics.bwh.harvard.edu/pph/) predicted that this variant is benign and therefore, no consequences of the polymorphism on the structure or function of P-glycoprotein arise. In the alignment output of PolyPhen, diverse amino acids were listed for the corresponding position in different species, indicating that this region was not highly conserved. Additionally, protein sequence alignment of five mammalian species using the program ClustalW (1.83) multiple sequence alignment from the EMBL toolbox (http://www.ebi.ac.uk/clustalw/index.html) gave no further evidence that the substitution had great impact on the protein because valine was also found at the corresponding position in the orthologous sequences for functional proteins of human, rat and mouse. Moreover, it has to be assumed that an exchange of a nonpolar amino acid with another nonpolar amino acid does not alter the structure of the protein significantly.

The simulated computer analysis gave no evidence that effects on structure or function of the *MDR1* protein have to be expected, but the c.3508A>G SNP might alter the expression level of *MDR1* mRNA and/or protein. For measurement of the *MDR1* expression, liver biopsies of four dogs, each two showing the genotype A/A or G/G, were analysed using qRT-PCR and the $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen, 2001). There was no significant difference between the relative mRNA expression levels of both genotypes. Natural variation in the individual *MDR1* expression might explain the difference between the mean values of genotypes A/A and G/G. However, a final conclusion about modification of mRNA expression caused by the newly detected SNP is not possible because the statistical power is restricted due to the limited number of samples.

Possible effects of the c.3508A>G SNP on expression levels, structure and stability of the canine *MDR1* mRNA, and thus resulting modifications of expression, structure or functionality of the protein seemed to be unlikely but could not be completely excluded in this study with simulated computer analysis and the measurement of *MDR1* expression in four dogs. Even the exchange of a nonpolar amino acid with another nonpolar amino acid might alter the stability of the protein structure by modifications in amount or character of bonds. Therefore, possible effects of the newly detected polymorphism in the canine *MDR1* gene on P-glycoprotein have yet to be clarified.

The results of this study show that the Elo breed is not affected by the mdr1-1Δ mutation in the fourth exon of the canine *MDR1* gene, but an A to G transition in exon 26 was found. Further polymorphisms in the canine *MDR1* gene which may have impact on P-glycoprotein can not be excluded. Sequence analyses of the complete cDNA and of the promotor region of the *MDR1* gene have to be performed to discover undetected polymorphisms, since this study was restricted to three exons of the canine *MDR1* gene whose orthologous human exonic sequences included three linked SNPs that were associated with decreased *MDR1* function and reduced mRNA and/or protein expression in some human tissues (Pauli-Magnus and Kroetz, 2004). The knowledge about functional polymorphisms in the canine *MDR1* gene is important because the degree of expression and the functionality of the *MDR1* protein can directly affect the therapeutic effectiveness of drugs. Modified function of P-glycoprotein can concern the absorption and elimination of drugs as well as accumulation in sensitive tissues like the brain after penetrating damaged blood-tissue barriers (Fromm, 2004). Moreover, the

substrate specificity may be modified by a mutation in the responsible region of the *MDR1* gene. To date, few drugs have been proved to be substrates of P-glycoprotein in dogs. In addition to ivermectin, neurotoxic adverse effects in Collies or in other mdr1-1Δ mutant dogs were described for doramectin (Yas-Natan et al., 2003), loperamide (Sartor et al., 2004), digoxin (Henik et al., 2006), moxidectin (Geyer et al., 2005a), dexamethasone (Mealey et al., 2007), vincristine, vinblastine and doxorubicin (Mealey et al., 2003). Due to the high degree of homology between species, the same drugs proved to be substrates for human or mouse P-glycoprotein are expected to be substrates for the canine *MDR1* protein as well (Mealey et al., 2007). Further studies about possible effects of polymorphisms in the *MDR1* gene on drug therapy in dogs including more precise knowledge about P-glycoprotein substrates and absorption as well as elimination of drugs are necessary. In addition to the protection of brain tissue from drugs, a functional P-glycoprotein in the blood-brain barrier plays an important role for the regulation of the hypothalamus-pituitary-adrenal (HPA) axis. Mealey et al. (2007) found that the HPA axis was suppressed in dogs with the mdr1-1Δ mutation. Elevated levels of cortisol can pass through the damaged blood-brain-barrier provoking a negative feedback in the hypothalamic-pituitary-adrenal axis and resulting in reduced production of hormones like cortisol. This may have negative effects for affected dogs during times of stress or illness. The physiological function of P-glycoprotein and possible negative effects of polymorphisms in the *MDR1* gene for the regulation of the hormone metabolism have to be researched into detail.

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Chapter 7

Summary

Summary

Silvia Fecht geb. Döpke (2007)

Analysis of the canine *MDR1* gene in the dog breed Elo

The objective of the present study was to analyse the canine *MDR1* gene in the dog breed Elo for the mdr1-1Δ mutation originating in the Old English Sheepdog breed as well as to search for functional polymorphisms in the gene.

A deletion mutation in the canine *MDR1* gene, mdr1-1Δ mutation, was found to be the cause of multiple drug sensitivity in several dog breeds from the Collie lineage including the Old English Sheepdog breed. A haplotype of four microsatellites containing this mdr1-1Δ mutation was conserved among affected breeds. The newly developed German dog breed Elo was supposed to be affected by the mdr1-1Δ mutation because four dogs with Old English Sheepdog ancestry whose *MDR1* genotypes were unknown participated in the foundation of the breed.

For the analysis whether the mutant mdr1-1Δ allele is segregating in the Elo population, 177 blood samples representative for the Elo breed on the basis of gene contributions by the Old English Sheepdog founder dogs were collected. At first, a polymerase chain reaction (PCR) based method with subsequent polyacrylamide gel electrophoresis was used for the detection of the mdr1-1Δ mutation. The mdr1-1Δ allele was not observed in the analysed sample of Elos. Subsequently, the probability that no mdr1-1Δ allele originating in the Old English Sheepdog breed is segregating in the Elo population was estimated at close to one ($1 - 3.68 \times 10^{-17} \approx 1$).

Furthermore, the *MDR1* flanking region of the sampled Elos was analysed using the four microsatellite markers *C14.866*, *REN144I15, REN103E18* and *G01506* to determine whether haplotypes of the Old English Sheepdog breed, which had been found to be associated with the mutant mdr1-1Δ allele, could be recovered in the Elo breed. The analysis revealed 35 different haplotypes in 177 Elo dogs. The "247-251-148-178-159" haplotype was the most frequent for the Elo breed with 31.87 %. The haplotype "247-255-148-173-159" which had previously been associated with the mutant mdr1-1Δ allele in Old English Sheepdogs was

detected in the sample of Elos with a frequency of 2.26%. Using a stepwise forward/backward regression analysis for the probability of this haplotype on the proportion of genes of the founder breeds, the Old English Sheepdog could be excluded as origin of this haplotype for the Elo breed. The *MDR1* flanking region could be traced back to the Japanese Spitz as one of the founder dog breeds of the Elo. Thus, we ruled out the introgression of the mdr1-1Δ mutation into the dog breed Elo through the Collie lineage.

Besides the mdr1-1Δ mutation associated with multiple drug sensitivity in several dog breeds, no further sequence variations in the canine *MDR1* gene have been described as yet in contrast to the numerous detected SNPs in the orthologous human *MDR1* (*ABCB1*) gene which have partly been associated with decreased amount or functionality of the *MDR1* protein (P-glycoprotein). To analyse whether functional polymorphisms exist in the exons 12, 21 and 26 of the canine *MDR1* gene, sequence analysis was performed in two Collies and six dogs of the Elo breed. The analysis revealed a nonsynonymous A to G exchange in exon 26 (c.3508A>G) which causes an amino acid substitution from methionine to valine. Subsequently, restriction fragment length polymorphism (RFLP) was used for the detection of this A to G transition in 88 Elo dogs and 65 dogs of different breeds. The presence of the G-allele in the analysed Elos with a frequency of 57.95% was mainly influenced by the founder breed Samoyed. Additionally, the mutant allele was found in Labrador Retrievers, Do-Khyis, Dalmatians, German Wirehaired Pointers, Hovawarts and Border Collies. In the analysis of protein alignments used for the prediction of possible impact of the amino acid substitution on the structure and function of the protein this sequence variant was predicted to be benign. Quantitative RT-PCR revealed no significant difference between the relative expression levels of *MDR1* in liver biopsies of each two Elos with the genotype A/A and G/G. Nevertheless, effects of the newly detected c.3508A>G SNP on expression, structure or functionality of *MDR1* mRNA and/or protein and the presence of further functional polymorphisms in the canine *MDR1* gene could not be completely excluded in this study.

Chapter 8

Erweiterte Zusammenfassung

Erweiterte Zusammenfassung

Silvia Fecht geb. Döpke (2007)

Analyse des caninen *MDR1*-Gens bei der Hunderasse Elo

Einleitung

Eine Deletionsmutation im vierten Exon des caninen *MDR1*(*multidrug resistance*)-Gens verursacht multiple Arzneimittelüberempfindlichkeit bei verschiedenen Hunderassen der Collie-Linie. Von dieser mdr1-1Δ-Mutation betroffene Hunderassen sind neben dem Collie zum Beispiel der Shetland Sheepdog, der Australian Shepherd und der Old English Sheepdog. Das bestehende Problem ist bei Tierärzten allgemein als „Ivermectin-Überempfindlichkeit" beim Collie bekannt. Hunde mit diesem genetischen Defekt zeigen schwere neurotoxische Nebenwirkungen, wenn sie mit bestimmten, strukturell zum Teil sehr unterschiedlichen Wirkstoffen behandelt werden. Vergiftungserscheinungen, die vom verabreichten Medikament und seiner Konzentration abhängen, variieren von milden Symptomen mit vermehrtem Speichelfluss und Desorientiertheit bis hin zu starken Auswirkungen mit Koma und Tod des Tieres. Obwohl sie für viele verschiedene Indikationen benutzt werden, sind alle diese Arzneimittel Substrate des vom *MDR1*-Gen codierten P-Glykoproteins. Die Funktion dieses ATP-abhängigen Arzneimittel-Transporters in der Blut-Hirn-Schranke und in anderen Blut-Gewebe-Schranken ist der Schutz der Organe vor dem Übertritt toxischer Substanzen aus der Blutbahn. Neben dem Gehirn ist P-Glykoprotein auch in der Leber, im Darm, in den Nieren, in der Plazenta und in den Hoden zu finden, wo es zur Begrenzung der Aufnahme toxischer Verbindungen aus dem Gastrointestinaltrakt und zur Unterstützung der Ausscheidung dieser Stoffe über die Leber, die Nieren und den Darm führt. P-Glykoprotein wird bei Hunden mit der mdr1-1Δ-Mutation nur stark verkürzt gebildet und verliert deshalb seine Funktionsfähigkeit, was zu einer erhöhten Durchlässigkeit der Blut-Hirn-Schranke mit der Folge von neurotoxischen Nebenwirkungen bei der Therapie mit bestimmten Arzneimitteln führt. Weiterhin kommt es durch die erhöhte Permeabilität der Blut-Hirn-Schranke zur verstärkten Passage von Cortisol, was zu einem negativen Feedback in der Hypothalamus-Hypophysen-Nebennieren-Achse führt und die verminderte Produktion der

entsprechenden Hormone zur Folge hat. Die mdr1-1Δ-Mutation befindet sich in einem für die Rassen der Collie-Linie konservierten Genomanschnitt. Die Vererbung des abstammungsidentischen mdr1-1Δ-Allels erfolgt monogen autosomal rezessiv. Die Prävalenz der Mutation variiert zwischen 0,6 und 64% abhängig von der betroffenen Rasse und deren geographischer Subpopulation. Durch sorgfältig geplante, züchterische Maßnahmen kann die mdr1-1Δ-Mutation teilweise oder ganz aus dem Genpool betroffener Rassen eliminiert werden. Die Entwicklung der Zuchtstrategie ist abhängig von der Prävalenz der Mutation in der jeweiligen Rasse und der Populationsgröße. Außerdem ist zu beachten, dass es mögliche, unerwünschte Nebeneffekte durch Allele von eng gekoppelten Genorten bei der Selektion auf das Wildtyp-MDR1-Allel geben kann.

Bis jetzt wurden in der Literatur noch keine weiteren Sequenzvarianten im *MDR1*-Gen des Hundes beschrieben. Im Gegensatz dazu wurden im orthologen humanen *ABCB1* (*MDR1*)-Gen zahlreiche Einzelnucleotidpolymorphismen (single nucleotide polymorphisms, SNPs) beschrieben, die teilweise mit Veränderungen der Menge oder Funktionalität des P-Glykoproteins in Zusammenhang gebracht wurden. Es wurden unter anderem drei SNPs [c.1236C>T, c.2677G>T/A und c.3435C>T] gefunden, zwischen denen ein Kopplungsungleichgewicht besteht. Des Weiteren wurde die synonyme Substitution c.3435C>T als funktioneller SNP identifiziert, der mit verringerter *MDR1*-Funktion sowie reduzierter mRNA- und Proteinexpression in einigen Geweben assoziiert war.

Der Elo ist eine junge, durch Kreuzung verschiedener Hunderassen begründete, deutsche Gesellschaftshunderasse. Die Zucht des Elos begann 1987 mit 16 Gründertieren aus den neun Rassen Eurasier, Old English Sheepdog, Chow-Chow, Samojede, Dalmatiner, Pekingese, Kleinspitz, Mittelspitz und Japanspitz. Zu den Gründertieren zählten vier Hunde mit Old English Sheepdog-Abstammung, von denen nicht bekannt ist, ob sie Träger des mdr1-1Δ-Allels waren.

Das Ziel dieser Dissertation ist es, das canine *MDR1*-Gen bei der Hunderasse Elo auf die mdr1-1Δ-Mutation, die über den Old English Sheepdog in die Population gelangt sein könnte, sowie auf funktionelle DNA-Polymorphismen zu untersuchen. Dazu wurde zuerst eine repräsentative Stichprobe von Hunden der Rasse Elo auf das Vorkommen der mdr1-1Δ-Mutation überprüft. Außerdem wurde bei diesen Hunden der die mdr1-1Δ-Mutation umgebende Genombereich untersucht, um zu überprüfen, inwieweit sich der in der Collie-

Linie konservierte Haplotyp in der Kreuzungspopulation Elo erhalten hat, und wie eindeutig diese Haplotypen für die Rassen der Collie-Linie sind. Anschließend sollte eine Sequenzanalyse weiterer Bereiche des caninen *MDR1*-Gens zeigen, ob dort funktionelle Polymorphismen gefunden werden können, die möglicherweise, wie beim Menschen, Einfluss auf die *MDR1*-Expression oder -Funktion haben.

Material und Methoden

Für die Untersuchung der mdr1-1Δ-Mutation wurden EDTA-Blutproben von 177 Hunden der Rasse Elo gesammelt, aus denen genomische DNA für die Analysen isoliert wurde. Für die Untersuchung des neu entdeckten Polymorphismus im Exon 26 des caninen *MDR1*-Gens konnten außerdem die genomische DNA von 65 Hunden verschiedener Rassen sowie vier Leberbiopsien von Elos benutzt werden.

Es standen die Zuchtbuchdaten für die gesamte Elo-Population vom Beginn der Zucht 1987 bis zum Ende des Jahres 2005 zur Verfügung, die mittels der Programme OPTI-MATE, Version 3.87 (Institut für Tierzucht und Vererbungsforschung, Tierärztliche Hochschule Hannover) und SAS, Version 9.1.3 (Statistical Analysis System, SAS Institute, Cary, NC, USA) ausgewertet wurden. Es wurden die Genanteile der verschiedenen Gründerrassen, Inzuchtkoeffizienten und Beiträge zur Inzucht durch die verschiedenen Gründertiere für die Elos der Stichprobe sowie für eine als aktuell definierte Population berechnet. Diese aktuelle Population umfasste 2973 Hunde, die zwischen 1994 und 2005 geboren wurden. Für die Rasse Old English Sheepdog wurde zusätzlich der jeweilige Genanteil der einzelnen vier Gründertiere mit Old English Sheepdog-Abstammung berechnet. Im Mittel betrug der Old English Sheepdog-Genanteil 20,79% in der aktuellen Population und 18,76% in der Stichprobe. Die reinrassige Old English Sheepdog-Hündin (Dam A) hatte den größten Einfluss aller vier Old English Sheepdogs auf die Elo-Population. Sie brachte im Mittel 17,42% der Genanteile in die aktuelle Population und 16,31% der Genanteile in die Stichprobe ein. Die drei anderen Hunde mit Old English Sheepdog-Abstammung brachten durchschnittlich jeweils weniger als 2% der Genanteile in die aktuelle Population ein. Die repräsentative Stichprobe von 177 Hunden der Rasse Elo spiegelte die Verteilung der Old English Sheepdog-Genanteile in der aktuellen Population wider.

Untersuchung des Vorkommens der mdr1-1Δ-Mutation beim Elo

Für die Untersuchung der DNA wurden vorher veröffentlichte Primer verwendet, die die mdr1-1Δ-Mutation umspannten. Die Auswertung erfolgte mittels PCR und Polyacrylamidgelelektrophorese anhand der Größe der PCR-Produkte. Beim Vorliegen des mutierten Allels wurde ein 144 bp großes Produkt und im Falle des Wildtyp-Allels ein 148 bp großes Produkt gebildet. Außerdem wurde die Wahrscheinlichkeit berechnet, dass kein mdr1-1Δ-Allel, welches vom Old English Sheepdog stammt, in der aktuellen Elo-Population segregiert. Die Berechnung basierte auf den Ergebnissen der Genotypisierung dieser 177 Hunde, dem mittleren Genanteil der Old English Sheepdog-Gründertiere in der Stichprobe und der Allelfrequenz in der Old English Sheepdog-Population.

Haplotypanalyse des MDR1 umgebenden Genomabschnitts beim Elo

Für die Haplotypanalyse des den *MDR1*-Locus umgebenden Genomabschnitts beim Elo wurden vier vorher veröffentlichte Mikrosatellitenmarker verwendet. Die Positionen der vier Marker und *MDR1* wurden in der Referenzsequenz des caninen Chromosoms (CFA) 14 aus der NCBI Datenbank (GenBank accession no. NC_006596) ermittelt. Entsprechend des Dog genome assembly 2.1 wurde die Reihenfolge wie folgt festgelegt: *C14.866-REN144I15-MDR1-REN103E18-G01506* mit den Positionen 15.6, 16.5, 16.6, 17.7 und 19.3 Mb auf CFA14. Alle Mikrosatellitenmarker wurden über PCR und Polyacrylamidgelelektrophorese ausgewertet. Die Allel- und Haplotypfrequenzen wurden unter Verwendung der Prozeduren ALLELE und HAPLOTYPE von SAS/Genetics, Version 9.1.3 (Statistical Analysis System, SAS Institute, Cary, NC, USA) ausgewertet. Zur Ermittlung des Ursprungs des Haplotyps „247-255-148-173-159“ wurden die Genanteile der verschiedenen Gründerrassen, Inzuchtkoeffizienten und Beiträge zur Inzucht durch die verschiedenen Gründertiere für die Hunde mit diesem Haplotyp im Vergleich zur aktuellen Population ausgewertet. Durch eine Regressionsanalyse mittels GLM von SAS, Version 9.1.3 wurde der Zusammenhang zwischen den Genanteilen der verschiedenen Gründerrassen bei den untersuchten Elos und der Wahrscheinlichkeit des Auftretens des Haplotyps „247-255-148-173-159“ statistisch ermittelt.

Suche nach funktionellen Polymorphismen in Exons 12, 21 und 26 des caninen MDR1-Gens

Neben der mdr1-1Δ-Mutation im vierten Exon des caninen *MDR1*-Gens wurden weitere Bereiche der DNA untersucht, um nach Sequenzvariationen zu suchen. Die genomische *MDR1* Referenzsequenz des Hundes (GenBank accession no. NC_006596.2) wurde mit der caninen *MDR1* Referenz-mRNA (GenBank accession no. NM_001003215.1) und der *ABCB1* (*MDR1*) Referenz-mRNA des Menschen (GenBank accession no. NM_000927.3) verglichen und Exon/Intron-Grenzen durch das mRNA-to-genomic alignment Programm Spidey (http://www.ncbi.nlm.nih.gov/IEB/Research/Ostell/Spidey/index.html) bestimmt. Es wurden die drei Exons 12, 21 und 26 des caninen *MDR1*-Gens (Dog genome assembly 2.1) ausgewählt, deren Sequenzen mit den orthologen Exons des menschlichen *ABCB1* (*MDR1*)-Gens übereinstimmten, welche die drei SNPs c.1236C>T, c.2677G>T/A und c.3435C>T enthielten. Flankierende Primer wurden mittels der Programme PRIMER3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) und Repeatmasker 3.1.0 (http://www.repeatmasker.org/cgi-bin/WEBRepeatMasker) entwickelt. Zunächst wurden die DNA-Sequenzen von sechs Elos und zwei Collies analysiert. Die PCR-Amplifikate wurden mit Hilfe des automatischen Sequenziergerätes MegaBACE 1000 sequenziert und die erhaltenen Sequenzen mit dem Programm Sequencher 4.7 ausgewertet. Anschließend erfolgte die Untersuchung des gefundenen SNPs im Exon 26 bei 88 Elos und 65 Hunden verschiedener Rassen mittels Restriktionfragment-Längenpolymorphismus (RFLP). Dabei schnitt das Restriktionsenzym *Bsg* I das PCR-Produkt beim Vorliegen eines G in der Erkennungssequenz. Die für den jeweiligen Genotyp typischen Fragmente wurden auf einem Agarosegel nach ihrer Größe aufgetrennt und ausgewertet. Die Auswertung der Genotypen des neuen SNPs erfolgte mit Hilfe der Prozedur ALLELE von SAS/Genetics, Version 9.1.3. Durch eine Regressionsanalyse mittels GLM von SAS, Version 9.1.3 wurde der Zusammenhang zwischen den Genanteilen der verschiedenen Gründerrassen bei den untersuchten Elos und der Verteilung der Allele A und G ausgewertet. Zur Simulation möglicher Effekte des Aminosäureaustauschs durch den SNP auf die Struktur oder Funktion des Proteins wurde das Programm PolyPhen (http://genetics.bwh.harvard.edu/pph/) benutzt. Der Vergleich der Aminosäuresequenz des Hundes mit den orthologen Sequenzen von Mensch, Maus, Ratte und Schaf erfolgte mittels des ClustalW (1.83) multiple sequence alignments der EMBL Toolbox (http://www.ebi.ac.uk/clustalw/index.html). Alle Referenz-

Sequenzen wurden der NCBI-Datenbank entnommen. Zur Überprüfung des Einflusses des gefundenen SNPs auf die Expression von *MDR1* wurden vier Leberbiopsien von je zwei Elos mit dem A/A- und dem G/G-Genotyp mittels qRT-PCR untersucht. Dabei wurde die *MDR1*-Transkript-spezifische Expression im Verhältnis zur *GAPDH*-Expression gemessen. Die Berechnung der relativen *MDR1*-Expression erfolgte mit Hilfe der 2-$\Delta\Delta C_T$-Methode, wobei die homozygote A/A-Probe als Kalibrator benutzt wurde. Die statistische Auswertung geschah mit Hilfe des t-Tests unter der Prozedur GLM von SAS, Version 9.1.3.

Ergebnisse

Untersuchung des Vorkommens der mdr1-1Δ-Mutation beim Elo

Bei den 177 Elos konnte kein mdr1-1Δ-Allel gefunden werden. Die Wahrscheinlichkeit, dass kein vom Old English Sheepdog stammendes mdr1-1Δ-Allel in der aktuellen Elo-Population segregiert, wurde auf annähernd Eins ($1 - 3{,}68 \times 10^{-17} \approx 1$) geschätzt.

Haplotypanalyse des MDR1 umgebenden Genomabschnitts beim Elo

Es wurden 35 verschiedene Haplotypen bei den 177 Elos der Stichprobe ermittelt, wovon 13 Haplotypen mit einer Frequenz von > 1% auftraten. Mit einer Frequenz von 31,87% war der Haplotyp „247-251-148-178-159“ am häufigsten zu beobachten. Es wurde ein Haplotyp „247-255-148-173-159“ mit einer Frequenz von 2,26% gefunden, der bei Hunden der Rasse Old English Sheepdog mit dem mutierten mdr1-1Δ-Allel assoziiert wurde. Bei der Untersuchung des Ursprungs des Haplotyps „247-255-148-173-159“ zeigte sich, dass der Genanteil der Old English Sheepdog-Gründertiere für die Elos mit diesem Haplotyp gering war, der Genanteil der Rassen Japanspitz, Kleinspitz und Pekingese jedoch relativ hoch im Vergleich zum Durchschnitt in der aktuellen Population. Zusätzlich trug der Japanspitz mit 11,16% bis 27,95% zu den Inzuchtkoeffizienten von sechs Elos mit dem Haplotyp „247-255-148-173-159“ überdurchschnittlich viel bei, während der Beitrag des Japanspitz zum Inzuchtkoeffizienten in der aktuellen Elo-Population (13,3%) mit 2,01% niedrig war. Mittels Regressionsanalyse wurden sechs von neun Gründerrassen als Ursprung des Haplotyps „247-255-148-173-159“ schrittweise ausgeschlossen. In der finalen Modellgleichung fanden nur noch die Genanteile der Rassen Kleinspitz, Pekingese und Japanspitz Berücksichtigung, während der Old English Sheepdog als Ursprung ausgeschlossen werden konnte. Schließlich

wurde die *MDR1* umgebende Region auf die Gründerrasse Japanspitz zurückgeführt, dessen Regressionskoeffizient mit p = 0,0314% signifikant war.

Suche nach funktionellen Polymorphismen in Exons 12, 21 und 26 des caninen MDR1-Gens
Die Sequenzanalyse der Exons 12, 21 und 26 bei sechs Elos und zwei Collies zeigte, dass keine Abweichungen in den Sequenzen der Exons 12 und 21 zu der genomischen Referenzsequenz des caninen *MDR1*-Gens bestehen. Es wurde ein Basenaustausch von A zu G im Exon 26 (c.3508A>G) in der genomischen DNA von vier der sechs Elos gefunden, wobei drei Hunde homozygot G/G und ein Hund heterozygot A/G waren. Zwei Elos und die zwei Collies waren homozygot A/A. Da die Boxer-Referenzsequenz (Dog genome assembly 2.1) an dieser Position ein A enthält, wurde diese Variante als Wildtyp definiert. Mit Hilfe des RFLPs wurde ermittelt, dass das G-Allel bei der untersuchten Stichprobe von 88 Elos mit einer Frequenz von 57,95% vorherrschend war. Außerdem konnte das G-Allel beim Labrador Retriever, Do-Khyi, Dalmatiner, Deutsch Drahthaar, Hovawart und Border Collie gefunden werden. Bei der anschließenden Ermittlung des Einflusses der Gründerrassen auf die Verteilung der Allele A und G bei den untersuchten Elos mittels Regressionsanalyse wurden schrittweise sieben von neun Gründerrassen ausgeschlossen. In der finalen Modellgleichung, die mit einem p-Wert von 0,0036 signifikant war, fanden nur noch die Genanteile der Rassen Samojede und Dalmatiner Berücksichtigung. Für die Gründerrasse Samojede ergab sich ein Regressionskoeffizient von +0,0201. Der Regressionskoeffizient für die Rasse Dalmatiner betrug –0,0519. Der c.3508A>G SNP im Exon 26 des caninen *MDR1*-Gens bewirkt einen Aminosäureaustausch von Methionin zu Valin an Position 1147 der Aminosäuresequenz des caninen *MDR1*. Die PolyPhen-Analyse ergab dennoch, dass diese Variante gutartig ist und somit keine weiteren Auswirkungen auf die Struktur oder Funktion des Proteins zu erwarten sind. Der Aminosäuresequenzvergleich mittels ClustalW zeigte, dass nur der Hund die Aminosäure Methionin an Position 1147 in der Wildtyp-Sequenz hat, während bei Mensch, Maus und Ratte an der entsprechenden Position in der Referenzsequenz Valin angegeben ist. Bei der Untersuchung der relativen *MDR1*-Expression in Leberbiopsien von vier Elos wurde kein signifikanter Unterschied (p = 0.069) zwischen dem A/A- (1.186 ± 0.121) und dem G/G-Genotyp (1.510 ± 0.084) festgestellt.

Diskussion

Der erste Aspekt dieser Arbeit war, das canine *MDR1*-Gen bei der Hunderasse Elo auf die mdr1-1Δ-Mutation zu untersuchen. Es wurde vermutet, dass der Elo das mdr1-1Δ-Allel tragen könnte, da zur Gründung der Rasse vier Hunde mit Old English Sheepdog-Abstammung verwendet wurden. Beim Old English Sheepdog wurde diese Mutation mehrfach, wenn auch in geringer Frequenz, nachgewiesen. Weiterhin gab es Studien, bei denen auch in anderen Kreuzungspopulationen mit Rassen der Collie-Linie das mdr1-1Δ-Allel nachgewiesen wurde. Für die Elos der untersuchten Stichprobe wurden etwas niedrigere Genanteile der Rasse Old English Sheepdog als in der aktuellen Population berechnet, was der Entwicklung des Anteils dieser Gründerrasse in der Elo-Population entsprach. Ansonsten spiegelte sich die Verteilung der Genanteile der Rasse Old English Sheepdog in der Stichprobe wider. Damit konnte ein für den Anteil an Old English Sheepdog-Genen repräsentativer Querschnitt der Elo-Population auf das Vorkommen der mdr1-1Δ-Mutation untersucht werden. Die Einkreuzung der mdr1-1Δ-Mutation bei der Rasse Elo durch die Collie-Linie konnte im Verlauf dieser Studie aus mehreren Gründen ausgeschlossen werden. Erstens konnte kein mdr1-1Δ-Allel in der für den Anteil an Old English Sheepdog-Genen repräsentativen Stichprobe gefunden werden. Zweitens ergab die Berechnung der Wahrscheinlichkeit basierend auf den Ergebnissen der Genotypisierung, dem durchschnittlichen Old English Sheepdog-Genanteil in der Stichprobe und der Frequenz des mdr1-1Δ-Allels in der Old English Sheepdog-Population annähernd Eins, dass kein mdr1-1Δ-Allel in der Elo-Population segregiert. Somit war ein unentdecktes Vorkommen des mutierten Allels in der Elo-Population sehr unwahrscheinlich. Drittens konnte zwar ein Haplotyp beim Elo gefunden werden, der beim Old English Sheepdog mit dem mutierten Allel assoziiert wurde, dieser kam jedoch beim Elo ohne die mdr1-1Δ-Mutation vor. Der Old English Sheepdog konnte daraufhin mittels Regressionsanalyse als Ursprung ausgeschlossen und der Haplotyp „247-255-148-173-159“ auf den Japanspitz als weitere Gründerrasse des Elo zurückgeführt werden. In der Literatur wurde dieser Haplotyp mit dem mdr1-1Δ-Allel assoziiert und eine starke Konservierung der den *MDR1*-Locus flankierenden Genomregion angenommen. Auf Grund der Ergebnisse dieser Studie ist es jedoch fraglich, ob diese genomische Region zwangsläufig mit dem mdr1-1Δ-Allel assoziiert ist. Weitere Studien der *MDR1* flankierenden Genomregion in von der mdr1-1Δ-Mutation betroffenen sowie nicht

betroffenen Rassen sind notwendig, um eine allgemeine Aussage über die Assoziation des mutierten mdr1-1Δ-Allels mit den in der Literatur beschriebenen Haplotypen machen zu können.

Der zweite Aspekt dieser Arbeit war, im caninen *MDR1*-Gen nach funktionellen DNA-Polymorphismen zu suchen, wie sie für das *ABCB1* (*MDR1*)-Gen des Menschen bereits beschrieben wurden und die zusätzlich zur mdr1-1Δ-Mutation Auswirkungen auf die Funktionsfähigkeit des P-Glykoproteins haben könnten. Bemerkenswerterweise war das mutierte G-Allel des neu gefunden c.3508A>G SNP im Exon 26 des caninen *MDR1*-Gens bei der Rasse Elo vorherrschend. Aus der Regressionsanalyse wurde gefolgert, dass das Auftreten des G-Allels bei den untersuchten Elos hauptsächlich durch die Gründerrasse Samojede beeinflusst wurde, wogegen die Gründertiere der Rasse Dalmatiner hauptsächlich das Vorkommen des A-Allels beeinflussten. Zwar wurde das mutierte Allel auch in anderen Rassen entdeckt, jedoch konnte auf Grund der geringen Anzahl untersuchter Hunde für andere Rassen keine Aussage über das Vorkommen des G-Allels gemacht werden. Weitere Untersuchungen zur Prävalenz in verschiedenen Hunderassen sind angezeigt, falls negative Auswirkungen des c3508A>G SNPs auf das *MDR1*-Protein nachgewiesen werden. Da der c.3508A>G SNP einen Aminosäureaustausch von Methionin zu Valin verursacht, sind Auswirkungen auf das P-Glykoprotein möglich. Da Valin an der entsprechenden Stelle bei funktionsfähigen Proteinen anderer Spezies wie Mensch, Maus und Ratte ebenfalls zu finden ist, ist es wahrscheinlich, dass das beim Hund im Falle des mutierten Allels gebildete Valin keine größeren Auswirkungen auf die Funktionsfähigkeit des P-Glykoproteins haben wird. Die Analyse mittels des Programms PolyPhen ergab ebenfalls, dass die Mutation gutartig ist. Außerdem wurden für die entsprechende Position beim Vergleich der Aminosäuresequenzen einige verschiedene Aminosäuren aufgelistet, so dass dieser Bereich nicht sehr konserviert erscheint. Ferner ist anzunehmen, dass der Aminosäureaustausch einer unpolaren Aminosäure mit einer anderen unpolaren Aminosäure keine großen Auswirkungen auf die Struktur des Proteins haben wird. Die Untersuchung der *MDR1*-Expression in Leberbiopsien von vier Elos ergab keinen Hinweis auf Auswirkungen des c3508A>G SNPs auf die mRNA-Expressionshöhe. Der geringe Unterschied zwischen den beiden Genotypen A/A und G/G ist durch die natürliche, individuelle Variation bei der Expression erklärbar. Es ist jedoch keine abschließende Aussage über eine mögliche Veränderung der *MDR1*-Expression durch den

entdeckten SNP möglich, da die statistische Aussagekraft auf Grund der geringen Probenanzahl eingeschränkt ist. Anhand der Ergebnisse der Computersimulation und der Untersuchung der *MDR1*-Expression in der Leber kann dennoch gesagt werden, dass Auswirkungen auf die Expressionshöhe, Stabilität, Struktur oder Funktion der caninen *MDR1*-mRNA und/oder des -Proteins unwahrscheinlich sind, aber noch nicht gänzlich ausgeschlossen werden können. Selbst der Austausch einer unpolaren mit einer anderen unpolaren Aminosäure kann zu Modifizierungen in der Menge und Art der Bindungen zwischen den Aminosäuren und damit zu Veränderungen der Stabilität der Proteinstruktur führen. Bemerkenswert ist auch, dass der gefundene c.3508A>G SNP nur eine Base stromabwärts vom c.3435C>T SNP der orthologen, menschlichen *MDR1*-Sequenz liegt, welcher mit verminderter *MDR1*-Funktion assoziiert wurde, obwohl dieser c.3435C>T SNP keinen Aminosäureaustausch und damit keine offensichtlichen Strukturveränderungen bewirkt.

Die Ergebnisse dieser Arbeit zeigen, dass zwar das Vorkommen der mdr1-1Δ-Mutation für die Rasse Elo ausgeschlossen werden kann, aber funktionelle Polymorphismen im caninen *MDR1*-Gen, welche Einfluss auf die Funktionsfähigkeit des P-Glykoproteins haben könnten, nicht auszuschließen sind. Zur Abklärung ist eine Sequenzanalyse der kompletten cDNA und der Promotorregion des caninen *MDR1*-Gens erforderlich, da sich diese Studie auf drei Exons des Gens beschränkte. Mutationen im caninen *MDR1*-Gen können sowohl die Schutzfunktion in Blut-Gewebeschranken als auch Absorptions- und Eliminationsfunktionen in anderen Organen negativ beeinflussen. Außerdem kann es auch zu einer Veränderung der Substratspezifität des P-Glykoproteins kommen. Daher ist die Kenntnis über das Vorkommen funktioneller Polymorphismen im caninen *MDR1*-Gen sowie deren Auswirkungen auf die Funktion des Proteins insbesondere für die Effektivität einer Arzneimitteltherapie wichtig. Weiterhin sind Untersuchungen der Konsequenzen für die Regulation der Hypothalamus-Hypophysen-Nebennieren-Achse im Falle eines beeinträchtigten P-Glykoproteins erforderlich.

Chapter 9

Appendix

Appendix

The label "Elo®" is protected by letters patent. For simplification, "Elo" is used as breed name in the dissertation.

Table 1 Gene contributions by founder dog breeds in the current Elo population (N = 2973 dogs born from 1994 until 2005)

Breed	N	Mean	S.D.	Minimum	Maximum
Old English sheepdog	2970	20.81	6.89	1.57	57.04
Eurasian dog	2970	55.49	14.87	3.13	78.10
Samoyed	214	9.52	9.20	1.57	50.00
Chow-Chow	2963	5.37	1.82	1.57	12.50
Pomeranian dog	1796	9.16	6.18	1.57	43.74
German Spitz	417	2.99	3.90	0.79	25.00
Dalmatian	134	5.54	3.33	1.57	25.00
Japanese Spitz	951	18.79	10.55	6.25	100.00
Pekingese	1796	9.16	6.18	1.57	43.75

Table 2 Primer sequences and annealing temperatures (T_a) for amplification of the microsatellite markers used in the haplotype analysis of the *MDR1* flanking region

Microsatellite	Forward primer (5' -> 3')	Reverse primer (5' -> 3')	T_a (°C)
C14.866	TGTCATAATAGTTGGAATGAC	TTAGAGCTTACTCATGATATCTG	56
REN144I15	GAGGACCTTGGAATGGCATA	TGCACTTGTAGGAAGCATGG	57
REN103E18	TGATTACTGCATACATAGC	AGACAAGAATAGCAACATC	52
G01506	TGGAGAACCAAATTGAGTCCT	GAAATCCACATTATATGAGGTTAAAC	57

Table 3 Marker allele frequencies in the 177 Elos genotyped

Microsatellite	Allele	Frequency (%)	Standard Error	95 % Confidence Limits	
C14.866	239	2.54	0.0083	0.0113	0.0424
	241	15.25	0.0203	0.1158	0.1921
	243	5.08	0.0120	0.0282	0.0763
	245	0.56	0.0040	0.0000	0.0141
	247	67.51	0.0268	0.6271	0.7260
	251	9.04	0.0165	0.0593	0.1243
REN144I15	251	72.88	0.0256	0.6808	0.7768
	255	9.60	0.0163	0.0650	0.1271
	257	6.21	0.0124	0.0395	0.0847
	259	9.60	0.0163	0.0650	0.1299
	261	1.69	0.0068	0.0056	0.0311
REN103E18	173	31.92	0.0269	0.2684	0.3729
	177	6.50	0.0138	0.0395	0.0932
	178	40.68	0.0263	0.3559	0.4548
	180	20.90	0.0217	0.1638	0.2514
G01506	151	5.08	0.0127	0.0282	0.0763
	153	1.13	0.0056	0.0028	0.0226
	155	6.50	0.0133	0.0395	0.0904
	157	9.32	0.0167	0.0621	0.1271
	159	57.63	0.0260	0.5282	0.6271
	161	7.06	0.0148	0.0424	0.1017
	165	13.28	0.0184	0.960	0.1695

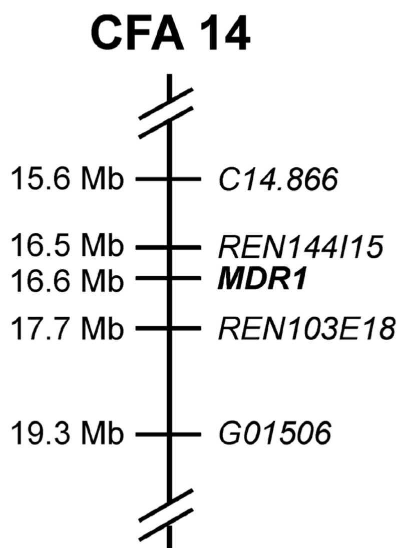

Figure 1 Map of the *MDR1* flanking region on CFA14. The order of loci was established on the basis of the distances between the markers (in Mb) according to the dog genome assembly 2.1.

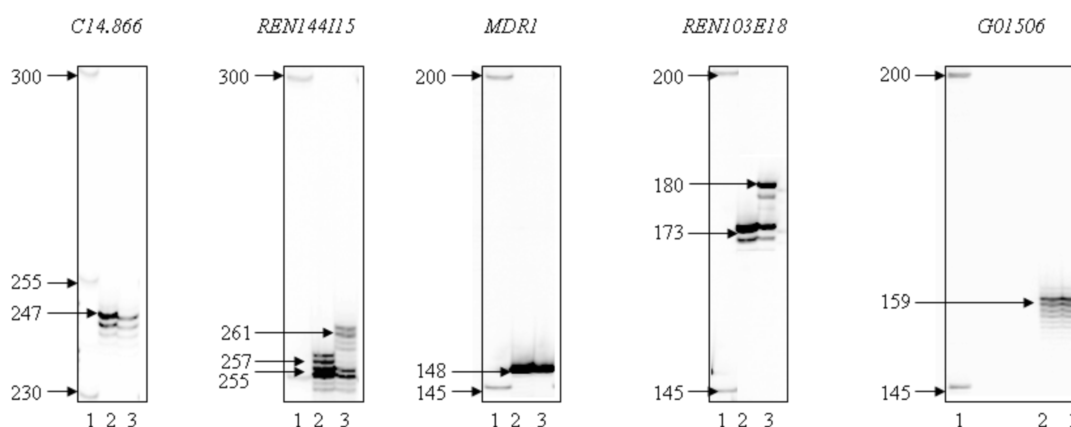

Figure 2 Microsatellite analysis. The PCR products produced bands of characteristic sizes (in bp) for each marker on the polyacrylamide gel. Both Elos genotyped were chosen exemplarily for the haplotype "247-255-148-173-159".

1: IRD-700- and IRD-800-labelled DNA-ladder respectively; 2: Elo No.13; 3: Elo No. 26

Laboratory paraphernalia

Equipment

Thermocycler

PTC-100™ Programmable Thermal Controller (MJ Research, Watertown, MA, USA)

PTC 100 ™ Peltier thermal Cycler (MJ Research, Watertown, MA, USA)

PTC 200 ™ Peltier thermal Cycler (MJ Research, Watertown, MA, USA)

Biometra TProfessional Thermocycler (Biometra, Göttingen, Germany)

Automated sequencer

LI-COR Gene Read IR 4200 DNA Analyzer (LI-COR Inc., Lincoln, NE, USA)

LI-COR Gene Read IR 4300 DNA Analyzer (LI-COR Inc., Lincoln, NE, USA)

MegaBACE 1000 (GE Healthcare, Freiburg, Germany)

7300 Real Time PCR System (Applied Biosystems, Darmstadt, Germany)

Centrifuges

Sigma centrifuge 4-15 (QIAGEN, Hilden, Germany)

Desk-centrifuge 5415D (Eppendorf AG, Hamburg, Germany)

Speed Vac® Plus (Savant Instruments, Farmingdale, NY, USA)

Electrophoresis chambers

OWL Seperation Systems, Portsmouth, NH, USA

Biometra, Göttingen, Germany

BioRad, München, Germany

Gel documentation system

BioDocAnalyze 312 nm (Biometra, Göttingen, Germany)

Pipettes

Multipette® plus (Eppendorf AG, Hamburg, Germany)

Pipetus®-akku (Hirschmann® Laborgeräte GmbH & Co.KG, Eberstadt, Germany)

Pipetman® (P2, P10, P20, P100, P200, P1000) (Gilson Medical Electronics S.A., Villiers-le-bel, France)

Pipettor, Multi 12 Channel (0.1 – 10 µl) (Micronic® systems, Lelystad, The Netherlands)

Impact® Pipettor 12-Channel (Matrix Technologies Corporation, Cheshire UK)

Impact® Pipettor 8-Channel (Matrix Technologies Corporation, Cheshire UK)

HAMILTON 8-Channel syringe (Hamilton Bonaduz AG, Bonaduz, Switzerland)

Others

Milli-Q® biocel water purification system (Millipore GmbH, Eschborn, Germany)

Incubator VT 5042 (Heraeus, Osterode, Germany)

UV-Illuminator 312 nm (Bachhofer, Reutlingen, Germany)

Centomat® R Desk-Shaker (B. Braun Melsungen AG, Melsungen, Germany)

Biophotometer (Eppendorf AG, Hamburg, Germany)

Kits

Isolation of DNA

QIAamp 96 DNA Blood Kit (QIAGEN, Hilden, Germany)

NucleoSpin Kit 96 Blood Quick Pure Kit (Macherey-Nagel, Düren, Germany)

Isolation of RNA

RNeasy 96 Universal TissueKit (QIAGEN, Hilden, Germany)

DNA purification

Montage PCR_{96} Cleanup Kit (Millipore GmbH, Eschborn, Germany)

MinElute® 96 UF Plate (QIAGEN, Hilden, Germany)

AutoSeq™ 96 Plate (GE Healthcare, Freiburg, Germany)

Sequencing

DYEnamic-ET-Terminator Cycle Sequencing Kit (GE Healthcare, Freiburg, Germany)

Genotyping

TaqMan® minor groove binding (MGB) probe (Applied Biosystems, Darmstadt, Germany)
SensiMix DNA Kit (Quantance Ltd, London, Great Britain)

Size standards

IRDye™ 700 or 800 (50bp - 350bp or 50bp - 700bp) (LI-COR Inc., Lincoln, NE, USA)
100 bp DNA Ladder (New England Biolabs, Frankfurt/Main, Germany)
1 kb DNA Ladder (New England Biolabs, Frankfurt/Main, Germany)

Primers

Primers were produced by MWG-Biotech AG, Ebersberg, Germany and biomers.net GmbH, Ulm, Germany.

Enzymes

PCR

SuperScript III Reverse Transcriptase (Invitrogen, Karlsruhe, Germany)
Taq-DNA-Polymerase 5 U/µl (Qbiogene/MP Biomedicals, Heidelberg, Germany)
Incubation Mix T.Pol with $MgCl_2$ [1.5 mM] (Qbiogene/MP Biomedicals, Heidelberg, Germany)
The polymerase was always used in the presence of incubation Mix T.Pol 10x buffer.

RFLP

The enzyme *Bsg* I (New England Biolabs, Frankfurt/Main, Germany) was used with the adequate 10x enzyme buffer.

Reagents and buffers

APS solution (10 %)

1 g APS

10 ml H_2O

Bromophenol blue solution

0.5 g bromophenol blue

10 ml 0.5 M EDTA solution

H_2O ad 50 ml

dNTP solution

100 µl dATP [100 mM]

100 µl dCTP [100 mM]

100 µl dGTP [100 mM]

100 µl dTTP [100 mM]

1600 µl H_2O

The concentration of each dNTP in the ready-to-use solution is 5 mM.

Gel solution (6%)

12.75 ml Urea/TBE solution (6%)

2.25 ml Rotiphorese® Gel 40 (38% acrylamide and 2% bisacrylamide)

95 µl APS solution (10%)

9.5 µl TEMED

Gel solution (4%)

13.5 ml Urea/TBE solution (4%)

1.5 ml Rotiphorese® Gel 40 (38% acrylamide and 2% bisacrylamide)

95 µl APS solution (10%)

9.5 µl TEMED

Loading buffer for gel electrophoresis

2 ml bromophenol blue solution

20 ml formamide

Loading buffer for agarose gels

EDTA, ph 8 [100mM]

Ficoll 400 20% (w/v)

Bromophenol blue 0.25% (w/v)

Xylencyanol 0.25% (w/v)

TBE-buffer (10x)

108 g Tris PUFFERAN® [121.14 M]

55 g boric acid [61.83 M]

7.44 g EDTA [372.24 M]

H_2O ad 1000 ml

pH 8.0

TBE-buffer (1x)

100 ml TBE-buffer (10x)

900 ml H_2O

Urea/TBE solution (6%)

425 g urea [60.06M]

250 ml H_2O

100 ml TBE-buffer (10x)

solubilise in a water bath at 65°C

H_2O ad 850 ml

Urea/TBE solution (4%)

425 g urea [60.06M]

300 ml H_2O

100 ml TBE-buffer (10x)

solubilise in a water bath at 65°C

H_2O ad 900 ml

Chemicals

Agarose (Invitrogen, Karlsruhe, Germany)

Ammonium persulfate (APS) ≥ 98 % (Sigma-Aldrich Chemie GmbH, Steinheim, Germany)

Boric acid ≥ 99.8 %, p.a. (Carl Roth GmbH & Co, Karlsruhe, Germany)

Bromophenol blue (Merck KgaA, Darmstadt, Germany)

dATP, dCTP, dGTP, dTTP > 98 % (Carl Roth GmbH & Co, Karlsruhe, Germany)

DMSO ≥ 99.5 %, p.a. (Carl Roth GmbH & Co, Karlsruhe, Germany)

dNTP-Mix (Qbiogene/MP Biomedicals, Heidelberg, Germany)

EDTA ≥ 99 %, p.a. (Carl Roth GmbH & Co, Karlsruhe, Germany)

Ethidium bromide (Carl Roth GmbH & Co, Karlsruhe, Germany)

Formamide ≥ 99.5 %, p.a. (Carl Roth GmbH & Co, Karlsruhe, Germany)

Paraffin (Merck KgaA, Darmstadt, Germany)

Rotiphorese®Gel40 (Carl Roth GmbH & Co, Karlsruhe, Germany)

Sephadex™ G-50 Superfine (GE Healthcare, Freiburg, Germany)

TEMED 99 %, p.a. (Carl Roth GmbH & Co, Karlsruhe, Germany)

Tris PUFFERAN® ≥ 99.9 %, p.a. (Carl Roth GmbH & Co, Karlsruhe, Germany)

Urea ≥ 99.5 %, p.a. (Carl Roth GmbH & Co, Karlsruhe, Germany)

Water was taken from the water purification system Milli-Q®

Consumables

Adhesive Film (-40°C bis +104°C) for PCR plates (nerbe plus, Winsen/Luhe, Germany)

Combitips® plus (Eppendorf AG, Hamburg, Germany)

MicroAmp™ Optical 96-well reaction plate (Applied Biosystems, Darmstadt, Germany)

MicroAmp™Optical Adhesive Film Kit (Applied Biosystems, Darmstadt, Germany)

PCR-Plate PP, nature, 96x0.2ml, skirted, RNase-, DNA- und pyrogenfree (nerbe plus, Winsen/Luhe, Germany)

Pipette tips 0.1 – 10 µl (7600) (Matrix Technologies Corporation, Lowell, USA)

Pipette tips 0.1 – 10 µl (K138.1), 0.1 – 10 µl (A407.1), 5 – 200 µl (7058.1) (Carl Roth GmbH & Co, Karlsruhe, Germany)

Reaction tubes 1.5 ml and 2.0 ml (nerbe plus GmbH, Winsen/Luhe, Germany)

Reaction tubes 10 and 50 ml (Falcon) (Renner, Darmstadt, Germany)

Thermo-fast 96 well plate, skirted (ABgene, Hamburg, Germany)

Software

BLAST, trace archive	http://www.ncbi.nlm.nih.gov http://www.ensembl.org
EMBL Toolbox	http://www.ebi.ac.uk/clustalw/index.html
OPTI-MATE, version 3.87	Wrede and Schmidt (2003), Institute of Animal Breeding and Genetics, University of Veterinary Medicine Hannover, Germany
Order of primers	MWG Biotech-AG, Ebersberg, Germany (https://ecom.mwgdna.com/register/index.tcl) biomers.net GmbH, Ulm, Germany (order@biomers.net)
Order of enzymes	http://www.neb.com/nebecomm/products/categories.asp
PolyPhen	http://genetics.bwh.harvard.edu/pph/
Primer design (PRIMER3)	http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi
Repeatmasker 3.1.0	http://www.repeatmasker.genome.washington.edu/
SAS/Genetics, version 9.1.3	Statistical Analysis System, SAS Institute, Cary, NC, USA
Sequencher 4.7	Gene Codes, Ann Arbor, MI, USA
Spidey	http://www.ncbi.nlm.nih.gov/IEB/Research/ Ostell/Spidey/index.html
SUN Ultra Enterprise 450	Sun Microsystems GmbH, Kirchheim-Heimstetten, Germany
SUN Fire V490	Sun Microsystems GmbH, Kirchheim-Heimstetten, Germany

Chapter 10

List of publications

List of publications

Journal articles

1. Fecht S., Wöhlke A., Hamann H., Distl O. (2007):
Analysis of the canine mdr1-1Δ mutation in the dog breed Elo
Journal of veterinary medicine. A, Physiology, pathology, clinical medicine, 54, 401-405.

2. Fecht S., Distl O. (2007):
Review of prevalence, genetic aspects and adverse effects of the mdr1-1Δ mutation in dogs
Übersichtsartikel zu Prävalenz, genetischen Aspekten und negativen Auswirkungen der mdr1-1Δ-Mutation des Hundes
Deutsche Tierärztliche Wochenschrift, accepted for publication

3. Fecht S., Wöhlke A., Distl O. (2007):
Haplotype analysis of the *MDR1* flanking region in the dog breed Elo
Berliner und Münchener Tierärztliche Wochenschrift, submitted for publication

Chapter 11

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