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# Investigation of Ivermectin Susceptibility in Kangal and Akbaş Dogs via MDR1 Gene Mutation

**Emine BAYDAN[1,a], Bengi ÇINAR KUL[2,b], Mustafa Yenal AKKURT[2,c,], Ebru YILDIRIM[3,d], Emre ARSLANBAŞ[4,e,*], Farah Gönül AYDIN[1,f]**

[1]Ankara University, Faculty of Veterinary Medicine, Department of Pharmacology and Toxicology, Ankara, Turkey.

[2]Ankara University, Faculty of Veterinary Medicine, Department of Genetics, Ankara, Turkey.

[3]Kırıkkale University, Faculty of Veterinary Medicine, Department of Pharmacology and Toxicology, Kırıkkale, Turkey.

[4]Aksaray University, Faculty of Veterinary Medicine, Department of Pharmacology and Toxicology, Aksaray, Turkey.

[a]ORCİD:0001-5459-8616;

[b]ORCID:0002-8955-0097;

[c]ORCID:0002-8139-0252;

[d]ORCID:0002-6289-0729;

[e]ORCID:0003-0030-7195;

[f]ORCID:0002-0068-2078.

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***Correspondence:** Emre Arslanbaş

Aksaray University, Faculty of Veterinary Medicine, Pharmacology and Toxicology Department, 68100, Aksaray/Turkey.

e-mail: emre.arslanbas@aksaray.edu.tr

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**Abstract:** This research aimed to investigate the sensitivity to the drug (ivermectin) in Kangal and Akbaş breed dogs, which are dog breeds native to Turkey, via the MDR1 gene mutation.

For the research, blood, hair and intraoral swap samples were taken from 30 Kangal and 20 Akbaş breed dogs (male-female mixed, adult) with ethical permission and approval forms. Kangal dog samples were taken from the dogs bred in the farms in Sivas center and Kangal village, and Akbaş dog samples were taken from the dogs bred in the farms in the Sivrihisar center, Ankara and Eskişehir regions. The samples taken were evaluated based on polymerase chain reaction (PCR) from the wool and subsequent two-way dideoxy chain termination reaction in the presence of 4 base farm deletions (c.296-299delAGAT) in the 4th exon of the ABCB1 gene that encodes the P-glycoprotein (P-gp) drug transporter protein.

According to the obtained DNA sequence results, the deletion of "AGAT" was not determined in any of the individuals screened. The results of this study, which is preliminary research, showed that Kangal and Akbaş breed dogs are safe in terms of sensitivity to drugs that are set to be absorbed and excreted by the P-gp pump, especially ivermectin. However, it would be useful to repeat the analysis of both breeds with more examples.

***Keywords:*** *Akbaş, Ivermectin, Kangal, MDR1 mutation, Susceptibility.*

## Kangal ve Akbaş Irkı Köpeklerde MDR1 Gen Mutasyonu Üzerinden İvermektine Duyarlılığın Araştırılması

**Özet:**Bu araştırmada, Türkiye'ye özgü iki farklı ırk köpekte (Kangal ve Akbaş) ivermektine duyarlılığın gen mutasyonu (MDR1) üzerinden belirlenmesi amaçlanmıştır.

Araştırma için, etik izni ve onam formu bulunan 30 adet Kangal ve 20 adet Akbaş ırkı köpekten (erkek dişi karışık, erişkin) kan, kıl ve ağız içi svap örneği alınmıştır. Kangal köpek örnekleri Sivas merkez ve Kangal köyünde yetiştirilen çiftliklerdeki köpeklerden, Akbaş köpek örnekleri ise, Sivrihisar merkez, Ankara ve Eskişehir bölgesindeki çiftliklerde yetiştirilen köpeklerden alınmıştır. Alınan örnekler P-glycoprotein (P-gp) drug transporter proteinini kodlayan ABCB1 (MDR1) geninin 4. ekzonunda 4 baz çiftlik delesyonun (c.296-299delAGAT) varlığı yönünden Polimeraz Zincir Reaksiyonu (PZR) ve takiben çift yönlü dideoksi zincir sonlanma reaksiyonuna göre değerlendirilmiştir.

Elde edilen DNA dizi sonuçlarına göre "AGAT" delesyonu taranan hiçbir köpekte belirlenmemiştir. Bir ön araştırma niteliğinde olan bu çalışmanın sonuçları, Kangal ve Akbaş ırkı köpeklerin başta ivermektin olmak üzere P-gp pompası ile emilme ve atılmaları ayarlanan ilaçlara karşı duyarlılık yönünden güvende olduklarını göstermiştir. Ancak, her iki ırka ait analizlerin daha çok örnekle tekrarlanması yararlı olacaktır.

***Anahtar Kelimeler:*** *Akbaş, Duyarlılık, İvermektin, Kangal, MDR1 mutasyonu.*

## Introduction

Kangal (Karabaş), Akbaş Çoban, Karayaka and Kars (Kafkas) Çoban dogs are some of the shepherd dogs native to Turkey. The most well-known and world-renowned of these dog breeds are the Kangal dogs, whose breed characteristics are best preserved (Yıldırım, 2012). Kangal dog is known as Anatolian Shepherd Dog abroad. In Turkey, although there are five different shepherd dog breeds, only three of which are registered (Kangal, Akbaş, Kars) and two of which are not yet registered (Sheep and Karaman shepherd Dogs). Kangal dog was registered as a breed by the Turkish Standards Institute with the number 11471 TS 12891 on 27.11.2002 (Yilmaz et al., 2015). In a study to understand the genetic relationship between Kangal dogs, Akbaş dogs and dogs from different regions of Eurasia, a 585 base pair segment of the mitochondrial DNA control region from Kangals and Akbaş was sequenced. When the sequences of Kangal and Akbaş dogs were compared with previous studies in dogs, it was reported that the results showed that Kangal and Akbaş dogs may have come from different maternal origins (Koban et al., 2009). In another study, the genetic diversity of Kangal dogs was analyzed using 100 canine microsatellites, and when the results were compared with Central Anatolian feral dogs, Akbaş dogs and Turkish greyhounds, it was reported that Kangal, Akbaş, Turkish greyhounds and feral dogs were significantly different from each other according to FST measurement (Altunok et al., 2005). Kangal dog is a dog breed that has an important place among Turkish shepherd dogs, which is compatible with the cold and less humid climatic conditions that Turks brought with them during their migration from Central Asia to Anatolia (Atasoy et al., 2005; Çoban et al., 2011; Yilmaz et al., 2012). It is grown all over Anatolia, especially Sivas (Ministry of Agriculture and Rural Affairs, 2009; Yıldırım, 2012). It has been reported that they were much more successful in Turkey in 1975 than other dog breeds that were trained for military purposes and bred for many years (Erol, 2008).

The natural habitat of Akbaş, another shepherd and guard dog breed unique to Turkey, is Sivrihisar, Afyon, Eskişehir, Polatlı and Ankara. There are two types of Akbaş, long and short hairy. They are adapted to the continental climate (Atasoy et al., 2011; Ministry of Agriculture and Rural Affairs, 2009). Akbaş dog was registered as a breed by the Turkish Standards Institute on 27.11.2002 with the number 11471 TS 12891 (Yilmaz et al., 2012). The Akbaş dog, unlike the Kangal dog, is insufficiently known in Turkey. However, its cultivation is becoming widespread day by day in some countries, especially in America. In a study conducted in America, Akbaş was found to be the most successful dog breed in herd protection (Tepeli et al., 2003).

Pharmacoepidemiology related to pharmacogenetics is an important sub-branch of pharmacology and examines the change in drug response due to genetic differences in individuals (Upadhyay et al., 2019). Dosage, type, age, gender and race are important factors in the effectiveness of a drug. Pharmacogenetics focuses on individual differences in responses to drugs at the same dose and determines individualized drugs and doses (Elewa and Awaisu, 2019).

Parasite control in animals is carried out with the use of appropriate anthelmintic drugs. Ivermectin, a drug of the avermectin group, is widely used due to its action on internal and external parasites, its safety index and wide effectiveness. However, in some dog breeds there is sensitivity even to low doses of this drug due to genetic mutation (MDR1/ABCB1) (Hürlimann et al., 2023; Mealey et al., 2023). In some dog breeds (Sheltie, Australia Shepherd), especially dogs of the Collie breed, ATP-binding cassette, subfamily B (MDR/TAP), member 1 gene (ABCB1), better known as the sensitivity of ivermectin, which is characterized by the mutation of the 4-base pair in the 4th exon (Löscher, 2023). This gene encodes the transporter P-glycoprotein (Permeability glycoprotein; P-gp), which acts as a transmembrane protein pump. These carrier proteins are expressed in various tissues such as tumor cells, brain capillaries, endothelial cells, intestinal epithelium cells, kidney proximal tubular epithelium cells, spinal cord, placenta and testicular cells, and strongly affect the absorption and excretion of certain drugs, especially ivermectin, which is a macrocyclic lactone (ML) (Erkens et al., 2009; Linardi and Natalini, 2006). These carrier proteins, especially those found in the blood-brain barrier, act as a pump for the removal of drug molecules from the central nervous system (CNS). Since the blood-brain barrier is not sufficiently developed (the pump does not work well) in susceptible breeds, there is sensitivity due to the release of gamma-aminobutyric acid (GABA) in the drug-related CNS. Dogs of the Collie breed are sensitive to even as low as 0.1 mg/kg of ivermectin compared to other dogs. Brain concentrations of ivermectin were found to be much higher in dogs with sensitivity and this was associated with limited P-gp expression (Janko and Geyer, 2013; Turner, 2005). Pgp deficiency causes excessive permeability of the blood-brain barrier and leads to the accumulation of ivermectin in the brain (Mealey et al., 2001). This situation results in the ivermectin acting on GABA-mediated chlorine channels and the death of the sensitive individual (Beckers et al., 2022). Regarding the MDR1 mutation in dog breeds, sensitivity to drugs such as acepromazine, butorphanol, cyclosporine, digoxin, doxorubicin, loperamide, vinblastine, vincristine is also mentioned, apart from ivermectin (Geyer and Janko, 2012). There are several studies to identify the MDR1 mutation in dogs (Beckers et al., 2022; Dekel et al., 2017; Kawabata et al., 2005; Tappin et al., 2012). Also, Gramer et al. (2011) examined the samples collected without separating the dogs into breeds such as Akbaş, Kangal, and Kars, in terms of MDR1 mutation under the name Anatolian Shepherd Dogs.

In this research, it was aimed to screen for 4-base deletion mutations in the 4th exon of the MDR1 gene (c.296_299delAGAT) in samples taken separately from registered Kangal and Akbaş dog breeds specific to Turkey by PCR/Sequence analysis method and to protect these breeds in pharmacogenetic context with conscious drug applications in case of possible mutations.

## Materials and Methods

**Obtaining biological samples and DNAs:** In the research, blood, hair and intraoral swab samples were taken from 30 Kangal bred in Sivas center and Kangal village and 20 Akbaş breed in Sivrihisar centers, Ankara and Eskişehir regions bred dogs (male-female mixed, adult), which were decided by the "Ankara University Animal Experiments Local Ethics Committee" (Decision; Date.10.02.2016, No. 2016-3-21), were used. Information about the pedigrees of the dogs was obtained from the owners of the animals, whose informed consent was signed for the study, and it was confirmed that they were not related for at least 3 generations, and their photographs were taken. Blood samples in the amount of 4-5 ml were taken from the *Vena saphena* or *Vena femoralis* of Kangal dogs into sterile needle-tipped vacuum EDTA tubes to represent each animal (n=30). Hair samples consisting of 5-6 hair bulbs were taken from interscapular area or intraoral swab samples were obtained from the buccal area to represent each animal, because of Akbaş dogs with an aggressive temperament or there is difficulty in taking a sample through a vein (n=20). The biological samples were transported to Ankara University Veterinary Medicine Faculty Genetics Department Molecular Genetics Laboratory under the cold chain (at +4°C). Blood samples were stored at -20°C, while swabs and hairs were at +4°C until the DNA isolation stage.

DNA isolation was performed with a commercial column-based kit (QIAamp DNA Mini Kit, cat. no. 51304, Qiagen, Germany), in accordance with the manufacturer's recommendations for all sample types. The integrity of the obtained DNAs was first checked by running under 90 volts electric current in 0.8% agarose gel electrophoresis stained with non-carcinogenic fluorescent dye (SafeView, Abmgood, cat. no. G108, Canada), then the amount and purity were determined by measuring with the spectrophotometric method Nanodrop (NanoDrop 2000, Thermo Fisher) device. After checking the purity and integrity of the DNAs, the DNA concentration of the isolates was diluted to 50 ng/µl and stored at -20°C until PCR processes.

**Polymerase chain reaction:** A pair of primer sets are designed to raise mutant and wild alleles in the elevation of the 4th exon of the MDR1 gene. Based on the reference genome sequence in primer design (Canis lupus familiaris breed boxer chromosome 14, CanFam3.1, whole genome shotgun sequence, GenBank accession code NC_006596.3:13725500-13727000 and Ensembl accession code ENSCAFG00845007972), "Primer3 (v.0.4.0)" (Koressaar and Remm, 2007) program was used. The primers used were indicated in Table 1.

In order to determine the binding conditions of the primers, graded (gradient) PCR was established at different temperatures (52-62°C range) and $MgCl_2$ conditions (1.5 and 2.5 mM). PCR procedures were performed with the Biorad C1000 Thermal Cycler located in the Molecular Genetics Laboratory, Department of Genetics, Veterinary Medicine Faculty, Ankara University. For this purpose, PCR 50 ng DNA, 1X buffer, 1.5-2.5 mM $MgCl_2$, dNTP (200 µM), forward and backward primers (5 pmol) and Taq DNA polymerase (1 U) were completed with $ddH_2O$ to a total volume of 25 µl. In order to determine the optimal binding temperature, 5 min at 95°C following the first denaturation, 30 sec at 95°C, 30 sec at 52-62°C range, 30 sec at 72°C were repeated at 39 cycles, and at 72°C 10 min final elongation was performed. PCR products were stained with non-carcinogenic fluorescent dye (SafeView, Abmgood, cat. no. G108, Canada), run under 120 volt electric current in 2% agar gel electrophoresis, then visualized under UV light with KODAK Gel Logic 200 device. The obtained PCR products were stored at -20°C until the purification and sequence analysis stage.

**Purification of PCR products and DNA Sequence Analysis:** PCR products were purified using a commercial kit (QIAquick PCR Purification Kit, cat. no 28104, Qiagen, Germany) before sequence analysis, and thus removing PCR residues.

The dideoxy chain termination reaction was established in accordance with the manufacturer's recommendations with the sequencing kit (BigDye™ Terminator v3.1 Cycle Sequencing Kit, cat. no 4337457) separately, with the forward and backward primers used in PCR at a concentration of 1.6 picomoles. Sequence PCR products were carried out in polyacrylamide gel capillary electrophoresis in ABI310 (Applied Biosystems, USA) device and bidirectional DNA sequences were obtained according to fluorescent light waves by laser system. Sequences (Hall, 1999) were aligned according to the reference sequence (Ensembl access code ENSCAFG00845007972) in the package program and consensus sequences were obtained. At the same time, electropherogram images related to sequences were checked with the eye in order to verify device readings.

## Results

**PCR results:** The results of the gradient PCR performed to determine the appropriate PCR conditions are given in Figure 1. Accordingly, the optimal condition was obtained at a concentration of 2.5 mM $MgCl_2$ and a binding temperature of 56°C (Figure 1). Then, PCR was applied to all samples at optimum temperature and $MgCl_2$ concentration (Figure 2), and after purification, dideoxy chain termination reaction was performed.

**Table 1.** MDR1 gene primers and expected amplicon size.

| Primary | Sequence, 5'-3' | Amplicon Size (bç) |
|---|---|---|
| Forward | CGCTATTCAAATTGGCTTG | 245 (wild) / 241 (mutant) |
| Backward | AATGAGGGCTAAACATCCTT | |

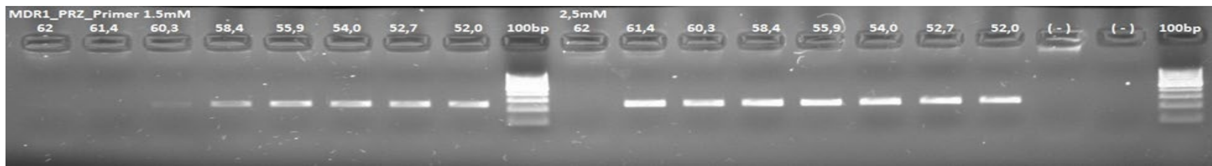


**Figure 1.** Gradient PCR experiment with concentration of 1.5 and 2.5 mM $MgCl_2$ and primary binding conditions at 62-52°C. Wells 9 and 20 show the 100 bp DNA ladder, and wells 18 and 19 show the negative control with water.

**DNA sequence analysis results:** The electrophoregram image and alignment results of some samples were presented in Figure 2 and Figure 3, respectively. Accordingly, when the DNAs obtained from Kangal and Akbaş breed dogs was bi-directional read backwards and forward with the primer and aligned according to the reference gene, it was determined that all the DNAs had a wild-type sequence and did not carry the corresponding mutation.

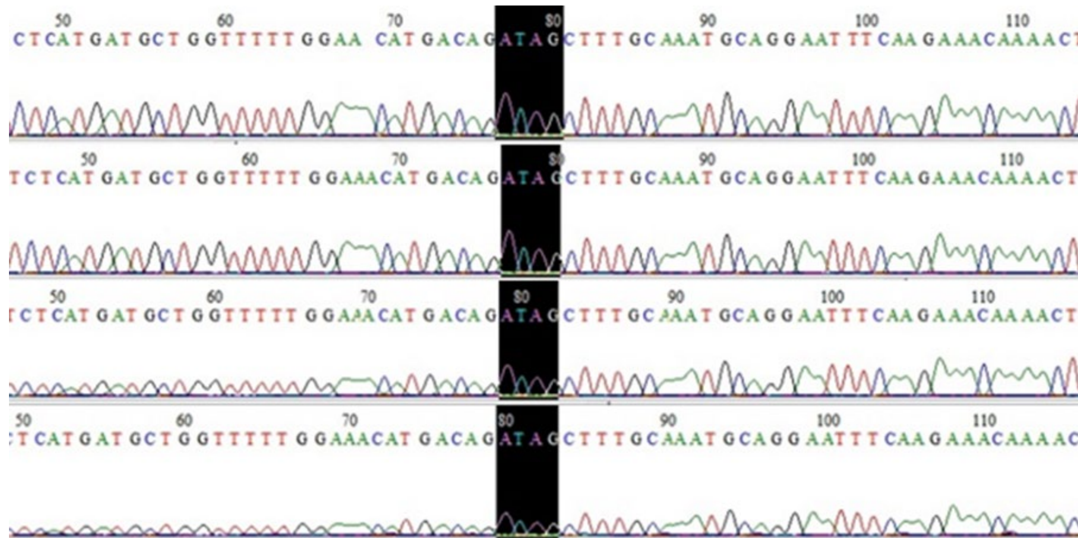


**Figure 2.** Corresponding electropherogram image of the MDR1 gene. The black area shows the mutation expected region.

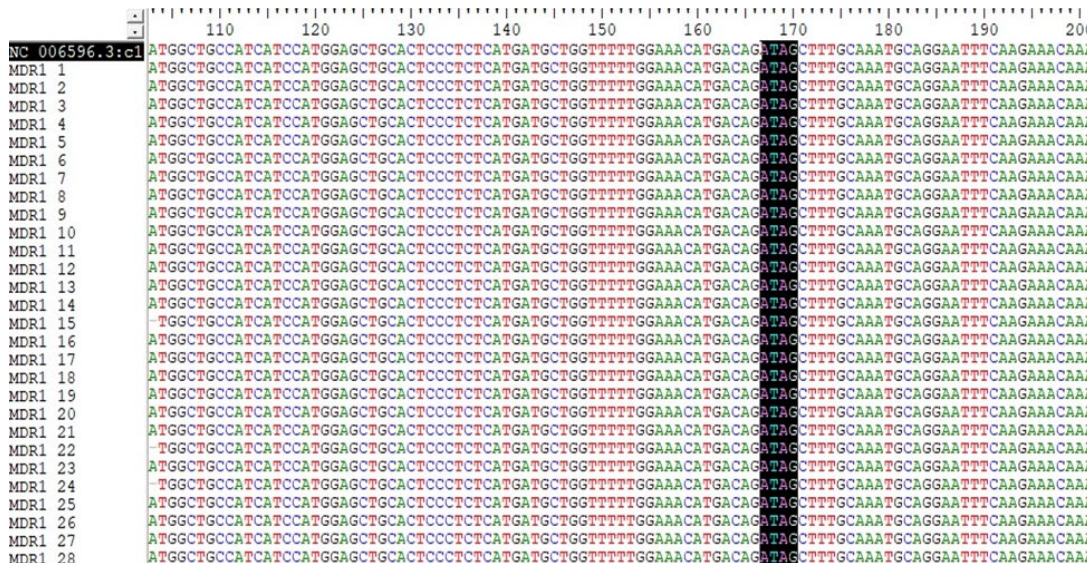


**Figure 3.** View of the sequences aligned in BioEdit according to reference sequence NC_006596.3.

## Discussion and Conclusion

Differences in efficacy or toxic effects of a drug between individuals are determined by genetic polymorphisms in drug-metabolizing enzymes, drug receptors, or drug transporters (Mealey and Meurs, 2008). While the importance of pharmacogenetics in humans is seriously emphasized, it is still in its infancy in veterinary medicine. The issue that draws attention to pharmacokinetics in veterinary medicine is the sensitivity to ivermectin in Collie

dogs and its association with the mutation in the MDR1 gene, which encodes the membrane carrier P-gp (Martinez et al., 2008). The most striking example of pharmacogenetics in dogs is the drug transporter P-gp. The P-gp protein, which is a part of the blood-brain barrier and acts as an important barrier to the passage of drugs to certain tissues, especially the brain, is encoded by the MDR1 gene (Mealey and Meurs, 2008). In addition to dogs, the MDR1 gene has also been recorded in mice, rats, ruminants, monkeys and humans (Asawakarn et al., 2012). The sensitivity/toxicity situation that has developed against ivermectin and some other drugs due to MDR1 mutation in some dog breeds (Geyer and Janko, 2012), especially collie dogs (Beckers et al., 2022), has caused a focus on the issue on a country-by-country basis and dog breeds to be screened from this point of view. In a scan conducted in Brazil, doxorubicin toxicity developed in a cross-breed dog and MDR1 mutation (c.296_299delAGAT) in the dog was shown as the cause (Monobe et al., 2013). MDR1 mutation was also detected in the analyses performed on Australian Shepherd, Collie, Shetland Sheepdog and Swiss White Shepherds in Belgium (Erkens et al., 2009). In a study conducted in Germany to determine the presence and frequency of the nt230 (del4) MDR1 mutation in dogs, the frequency of homozygous mutated genotypes in 1500 dogs scanned was highest in the Collie breed (33.0%), followed by the Australian Shepherd Dog (6.9%) and Shetland Sheepdog (5.7%). Screening revealed heterozygosity for the mutant MDR1 (-) allele in Waller dogs (37%) and Old English Sheepdogs (12.5%) (Geyer et al, 2005). In Brazil, 103 Collies, 77 Border Collies, 76 Shetland Shepherd Dogs, 20 Old English Shepherd Dogs, 55 German Shepherd Dogs, 16 Australian Shepherd Dogs and 53 Whippet Dogs were screened for MDR1 mutation. As a result of the study, the frequency of heterozygous mutated genotype MDR1 (+/-) in Collies, Australian Shepherds and Shetland Shepherds was determined as 50.5%, 31.3% and 15.8%. The presence of homozygous mutated genotype MDR1 (-/-) has been reported to be 35.9% only in Collies (Monobe et al, 2015). In a retrospective study covering a 5-year period in Italy, the detection status of the MDR1 mutated allele in dog populations (811 dogs) was attempted to be determined. At the end of the research, it was reported that the presence of mutated alleles was detected in 9 of 31 breeds (Rough Collie, Smooth Collie, Border Collie, Bearded Collie, Shetland Shepherd Dog, Australian Shepherd Dog, White Swiss Shepherd Dog, Old English Shepherd Dog, Whippet and also in hybrids) (Marelli et al., 2020). In a study conducted in Portugal, among 105 dogs of different breeds scanned (23 Barbado da Terceira, 10 Cão da Serra d'Aires, 55 carrying mutations, Australian Shepperd, Border Collie etc., 17 Labrador Retriever, Jack Russel) and it has been reported that the presence of mutation was detected for the first time in the Terceira breed in Barbado (Barroso et al, 2022). Similarly, in a study in Thailand in which MDR1 gene mutation was tried to be determined in 263 dogs belonging to eight purebred dog breeds using the allele-specific multiplex PCR method and direct DNA sequencing, the mutant allelic frequency was determined for Australian Shepherd Dogs, Shetland Shepherd Dogs and Old English Shepherd Dogs were 57.14%, 12.82%, 11.28% and 8.33%, respectively (Lerdkrai and Phungphosop, 2021).

On the other hand, MDR1 mutation was not found in Uruguay Cimmaron dogs by Rosa Gagliardi et al. (2013), German Shepherd, Doberman, Border Collie and Greyhound breed dogs (total 95 dogs) by Rosa Gagliardi et al. (2015) and Bearded Collies and German Shepherds breed dogs in the screenings of 92 dogs in Belgium by Erkens et al. (2009).

In population scans, the percentage of heterozygosity or allele frequency is usually taken into account. In the case of heterozygosity, which means the coexistence of a mutant allele and a wild allele, P-gp deficiency is less severe than homozygous mutants and may be clinically overlooked. Especially in homozygous dogs (MDR1 mutant/mutant), the passage of Ppg-dependent drugs to the brain is greater than in heterozygous dogs (MDR1 mutant/normal). Mealey et al. (2001) reported that there is a relationship between ivermectin sensitivity and deletion mutation of the MDR1 gene and that while dogs homozygous for the deletion mutation show sensitivity to ivermectin, homozygous normal or heterozygous dogs do not show increased sensitivity to ivermectin. In a study by Mealey and Meurs (2008), the presence of mutation was investigated in 5368 dogs, including 140 pure breeds and their hybrids, and the dog breeds with the highest percentage of heterozygosity were determined as Longhaired Whippet (58%) and Collie (42%). While the Collie breed dogs were represented by 1424 dogs, the Longhaired whippet breed was evaluated with only 24 dogs in this study, when the mutant allele frequency in the population was calculated by including homozygous mutants, values of 29% and 56% were obtained, respectively and high heterozygosity value was interpreted to be close inbreeding and working with less samples. In this study, 10 Akbaş dogs were also examined and no mutations were found. In another study by Gramer et al. (2011), the highest allelic frequency value was found to be 59% in Collies, 45% in Longhaired whippet and 30% in Shetland sheepdog breed in 7378 dogs consisting of 106 pure breeds and hybrids. However, the MDR1 nt230(del4) mutation has not been determined in some breeds, including the Anatolian shepherd dog breed, and it has been found that it is much rarer in some breeds.

In this study, which was carried out to determine the 4-base deletion mutation in the 4th exon of the MDR1 gene (c.296_299delAGAT) in blood, hair and intraoral swap samples of 30 Kangal and 20 Akbaş dog breeds native to Turkey by PCR/sequence analysis method, a mutation status could not be determined in either Akbaş or Kangal breed dogs (Figure 2 and 3). In the study conducted on Uruguayan Cimarron dogs, a relationship was established between not applying to the clinic for ivermectin toxicity and the absence of mutations in these breeds (Gagliardi et al., 2013). Since there is no retrospective study on the toxicity or sensitivity of ivermectin related to these breeds native to Turkey, such a relationship could not be established. However, it is thought that increasing the number of samples and increasing the frequency of controls will be useful in terms of clarifying the mutation status in special breeds.

## Conflict of Interest

The authors stated that they did not have anyreal, potential or perceived conflict of interest.

## Ethical Approval

This study was approved by the Ankara University Animal Experiments Local Ethics Committee (10.02.2016, 2016-3-21 Number Ethics Committee Decision). In addition, the authors declared that Research and Publication Ethical rules were followed.

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## Author Contributions

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Analysis and / or Interpretation: EB, BÇK, MYA, EY
Literature Review: EB, BÇK, EY, EA
Writing the Article: EB
Critical Review: BÇK, EY, EA

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