PhD THESIS SUMMARY

Research concerning canine inherited neurological disorders and molecular techniques used for the diagnosis of canine degenerative myelopathy

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I. Introduction

According to the OMIA (Online Mendelian Inheritance in Animals) database, there are 418 Mendelian inherited disorders in dogs, of which 355 have their causal variation identified (Nicholas & Tammen, 1995). Genomic studies provide information on the hereditary traits of diseases and the possibility of developing and identifying markers for diagnosing carriers, and subsequently enabling intervention through selective breeding to prevent the emergence of these conditions (Shearin & Ostrander, 2010). Additionally, there are multiple conditions in dogs that can serve as genetic models for future research on human diseases, thus facilitating the description of genomic mutations in both humans and dogs (Shaffer, 2019).

A large number of hereditary neurological disorders have been documented in dogs. These conditions encompass congenital, neonatal, late-onset, as well as progressive and non-progressive disorders. Consequently, any dog displaying neurological signs may potentially have an underlying hereditary condition. In cases where the mutation follows a recessive pattern, clinically affected animals are born only when consanguinity occurs, resulting in the inheritance of two identical copies of the mutation (one from each parent, who are healthy but carriers of the gene). Therefore, certain hereditary diseases are generally associated with specific breeds or closely related breeds due to shared genetic background (Mellersh, 2014).

Canine degenerative myelopathy (CDM) has been known for nearly 50 years as a disease that manifests in adult dogs and is characterized by neurodegenerative processes of the spinal cord. The disease is characterized by progressive deterioration of motor functions. It was first described in the German Shepherd breed in 1973 (Averill, 1973). The genetic variation responsible for the onset of the condition was identified in 2009 (Awano et al., 2009), as a transition from G to A (*c.118G>A*) in exon 2 of the *SOD1* gene (superoxide dismutase 1).

Given that most genetic variations have low frequencies, it can be challenging for any veterinarian to identify and possess the expertise to diagnose the wide range of hereditary disorders. This underscores the importance of continuous medical education and the presence of veterinary specialists with specific expertise in genetic counseling. Furthermore, emphasis is placed on the importance of comprehensive screening and diagnostic techniques for these inherited conditions.

Work hypothesis

Within the literature review section, the first chapter aimed to identify the molecular genetic techniques used in diagnosing Mendelian inherited genetic diseases in dogs in order to explore the technical and logistical requirements. Subsequently,

inherited neurological conditions with Mendelian pattern of transmission and known causal mutations were summarized to observe the applicability of these techniques. Among these conditions, canine degenerative myelopathy (CDM) stood out as the most well-documented hereditary disorder of the nervous system in dogs, thus, this condition was extensively described.

The first part of the original research aimed to classify canine inherited neurological conditions with Mendelian pattern of transmission into the modified ABC system and to identify their pathological and genetic significance.

The second part of the own research aimed to identify genetically determined diseases in dogs recorded within the Discipline of Morphopathology and Necropsy Diagnosis at FMV Cluj-Napoca over a period of 10 years.

The research in the final part of the thesis is dedicated to canine degenerative myelopathy. Regarding CDM, the goal was to establish and validate an efficient working protocol for the PCR technique by testing two different working protocols for identifying the *SOD1* gene and then identifying possible mutations using the RFLP technique. Furthermore, extensive testing of purebred dogs in Romania was conducted to estimate the prevalence of CDM and finally, the validation of genotyping was done via Sanger sequencing.

Objectives

1. Analysis of canine inherited neurological disorders with Mendelian pattern of transmission and known causal mutations, and characterizing them based on the level of documentation of the mutations;

2. Conducting a retrospective study to identify genetically diagnosed diseases in dogs at the discipline of Morphopathology and Necropsy Diagnosis, belonging to FMV Cluj-Napoca;

3. Testing various working protocols for molecular genetic techniques applied in the diagnosis of canine degenerative myelopathy (PCR-RFLP);

4. Molecular monitoring of the prevalence of the *SOD1:c.118G>A* mutation associated with canine degenerative myelopathy in purebred dogs in Romania;

5. Validation of genotyping via Sanger sequencing;

6. Presenting a series of case studies typologies in order to highlight the importance of genetic testing for CDM in a clinical context.

II. PERSONAL CONTRIBUTIONS

1. Analysis of neurological disorders with mendelian inheritance and known causal mutation, and their characterization in accordance with the strength of evidence related to the variant

The aim of the current research was to identify repetitive patterns within canine inherited neurological disorders with Mendelian pattern of transmission as described in the medical literature and to classify these conditions based on the level of documentation.

The pursued objectives were:

- Identifying dog breeds predisposed to developing inherited neurological disorders;
- Determining the frequency of mutation types involved in inherited neurological disorders;
- Characterizing the level of documentation of inherited neurological disorders based on functional and clinical evaluation using the modified ABC classification system utilized in classifying specific human mutations.

Conclusions:

Dog breeds which showed a predisposition to developing such disorders include Golden Retrievers, with 6 documented inherited neurological disorders with known causal mutations, and Belgian Shepherds, with 5 documented inherited neurological disorders with known causal mutations.

The most documented and widespread hereditary neurological disorder is canine degenerative myelopathy, documented in over 120 dog breeds.

The most common types of mutations for inherited neurological disorders in dogs are missense mutations (n = 46, 41%) and frameshift deletions (n = 21, 19%), according to Fig. 1.

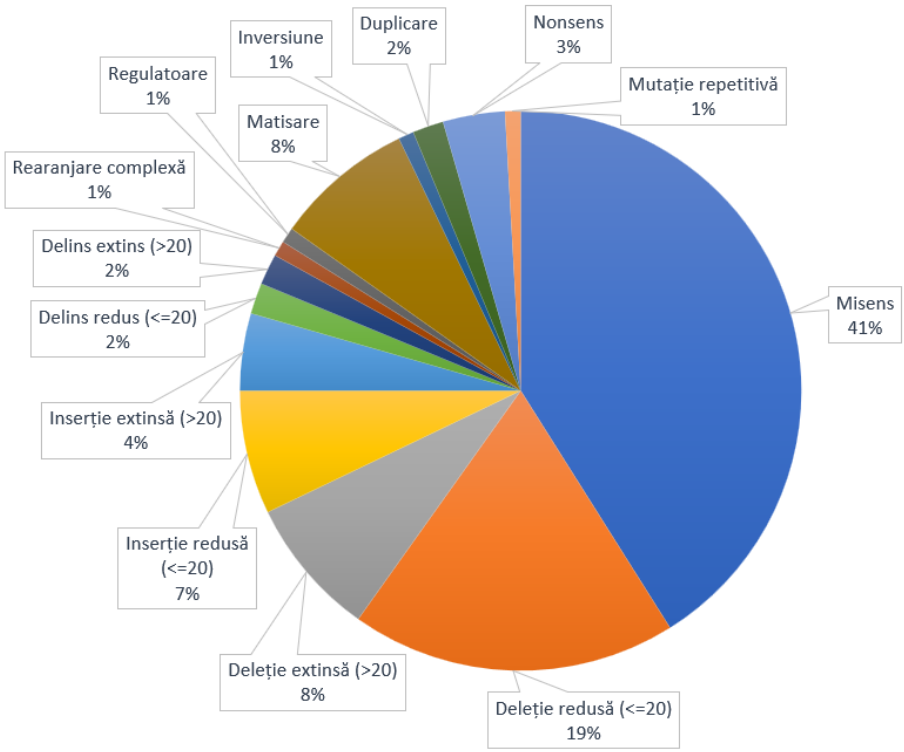

Fig. 1 – Distribution of variant types specific to neurological inherted disorders

The evaluation in the modified ABC system identified that 16 of the variants were classified as pathogenic variants, while the remaining 96 were classified as likely pathogenic variants.

2. Retrospective study on the canine genetic disorders identified via necropsy

The aim of the present research was the practical approach of genetic cases by identifying genetically diagnosed diseases in dogs based on the study of case records registered within the Discipline of Morphopathology and Necropsy Diagnosis at the Faculty of Veterinary Medicine Cluj-Napoca.

The pursued objectives were:

- Analysis of the records of the Discipline of Morphopathology and Necropsy Diagnosis over a period of 10 years (January 2012 – January 2022).
- Identification of canine cases with diagnoses that could have a genetic etiology and their classification based on the anatomical systems involved.

Conclusions:

A total of 206 dogs with at least one condition that could be attributed as genetic were identified. Cases with neoplasms, infectious or parasitic diseases, inflammatory disorders, traumas, intoxications, tissue samples, biopsies, cytological aspirates, and cases destined solely for incineration were excluded from the study.

Within the genetic disorders of the digestive system category, 73 canine cases were identified with the following pathologies: gastric dilatation-volvulus (n = 62), megaesophagus (n = 4), palatoschisis (n = 3), transdiaphragmatic hernia (n = 2), cheilopalatoschisis (n = 1), and intestinal stenosis (n = 1).

In the category of genetic disorders of the cardiovascular system, 115 canine cases were identified with the following pathologies: dilated cardiomyopathy (n = 63), hypertrophic cardiomyopathy (n = 35), subvalvular aortic stenosis (n = 4), aortic valve stenosis (n = 3), atrial septal defect (n = 2), ventricular septal defect (n = 2), persistent ductus arteriosus (n = 2), mitral valve dysplasia, pulmonary arterial trunk stenosis (n = 2), dextroposition of the aorta (n = 1), and concomitant presence of atrial canal, ventricular septal defect, and atrial septal defect (n = 1).

In the category of genetic disorders of the nervous system, 10 canine cases of internal hydrocephalus were identified.

Regarding disorders of the musculoskeletal system, 5 canine cases of hip dysplasia were identified.

In the category of genetic genitourinary disorders, 8 canine cases of cryptorchid testicles and 3 cases of renal dysplasia were identified.

It is recommended for the veterinarians to inform and raise awareness among owners regarding the genetic origin of diseases identified in this study, implement genetic prophylaxis measures, and perform molecular genetic tests for screening purposes or for differential diagnosis.

3. Comparison of molecular genetic techniques applied in the diagnosis of canine degenerative myelopathy

The current study aimed to conduct preliminary research to identify methods and techniques of molecular genetics applied for the diagnosis of canine degenerative myelopathy (CDM).

The main objectives of this study were to optimize the PCR protocol and the Restriction Fragment Length Polymorphism (RFLP) technique for the identification of the *C.118G>A* mutation of the *SOD1* gene. Additionally, the study aimed to identify the *C.118G>A* mutation of the *SOD1* gene through PCR-RFLP technique in the Carpathian Shepherd Dog breed.

Conclusions:

The current study is the first to focus on the molecular monitoring of the *c.118G>A* variant of the *SOD1* gene associated with canine degenerative myelopathy in the Carpathian Shepherd Dog breed. The PCR-RFLP technique proved to be sensitive and specific, demonstrating its utility in this regard. Due to the clearer amplicons resulting from the application of the second incubation protocol (adapted according to Holder et al., 2014), it was decided to use this protocol for testing the following 19 samples. Additionally, based on the obtained results, MyTaq Red polymerase was chosen for sample testing. The results indicated that the tested individuals belonging to the Carpathian Shepherd Dog breed were homozygous for the normal alleles (G/G), with no mutant allele (A) identified.

4. Molecular surveillance of canine degenerative myelopathy in breeding kennels from Romania

The aim of this study is to estimate the prevalence of the *SOD1:c.118G>A* mutation characteristic of canine degenerative myelopathy in purebred dogs in Romania.

To achieve the proposed aim, the research had the following objectives:

- Collection of buccal swab samples from purebred dogs (certified through pedigree);
- Processing of collected samples through PCR-RFLP;
- Classification of individuals based on genotype, using the obtained results;
- Estimation of the prevalence of the mutant allele in purebred dogs;
- Highlighting the importance of monitoring the mutation associated with canine degenerative myelopathy in clinical and cynological contexts;
- Comparison of the obtained results with those from the scientific literature;
- Development of recommendations for clinicians and dog breeders.

Conclusions:

The current study provides significant data regarding the occurrence of the *SOD1:c.118G>A* mutation in 28 kennels from Romania. To our knowledge, this study reports the first screening of dogs from the Romanian Shepherd, Caucasian Shepherd, and Romanian Mioritic Shepherd breeds for canine degenerative myelopathy.

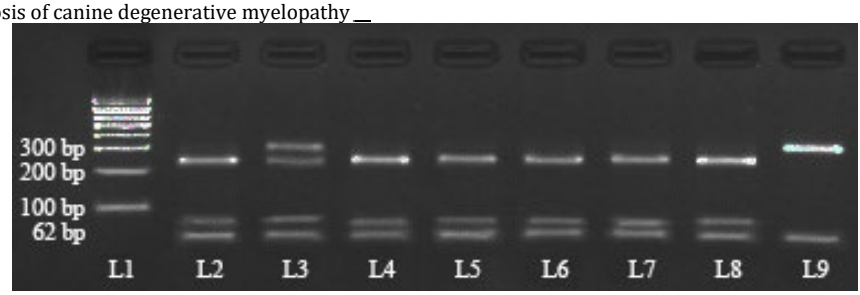

Fig. 2. The results of agarose gel electrophoresis following PCR-RFLP analysis.

Legend: L1 - 100 bp DNA ladder. Samples L2, L4, L5, L6, L7, L8 correspond to the G/G genotype (230 bp and 62 bp); Sample L3 corresponds to the A/G genotype (292 bp, 230 bp, and 62 bp); Sample L9 corresponds to the AA genotype (292 bp).

The success rate of DNA isolation and amplification (PCR-RFLP) was 100%, demonstrating that oral swabs were good sources for genetic testing of degenerative myelopathy. The genotypes G/G, A/G, and A/A can be observed in Fig. 2, which presents the results obtained following PCR-RFLP analysis. Out of the 230 tested dogs, 204 were healthy homozygotes (G/G), 16 were heterozygotes (A/G), and 10 were homozygotes for the mutant allele (A/A). The mutant allele (A) was identified in 6 out of the 26 tested breeds. The frequency of the mutant allele (A) associated with canine degenerative myelopathy for the 230 samples was 0.0783. Segregation of results by breeds indicated a frequency of 1 for Wire Fox Terrier, 0.2500 for Romanian Mioritic Shepherd, 0.2000 for German Shepherd, 0.0833 for Rottweilers, and 0.0313 for Belgian Shepherd. The *SOD1:c.118G>A* variant was identified for the first time in the Romanian Mioritic Shepherd breed. However, the mutation was not identified in specimens from the Romanian Shepherd and Caucasian Shepherd breeds included in this study.

5. Validation of genotyping via Sanger sequencing

The purpose of this study is to validate the previously presented results by comparing the electropherograms with the reference sequence for the canine *SOD1* gene.

The pursued objectives were as follows:

- Amplification, purification, and Sanger sequencing of two samples corresponding to each genotype (G/G, A/G, A/A) specific to canine degenerative myelopathy.
- Quality analysis, visualization, and processing of the resulting sequences using Geneious 4.8.5 software.

Conclusion:

Sequencing of the six DNA samples obtained from dogs with all three genotype variants (G/G, A/G, and A/A) and analysis of the resulting electropherograms confirmed the results obtained through the PCR-RFLP technique, as depicted in Fig. 3.

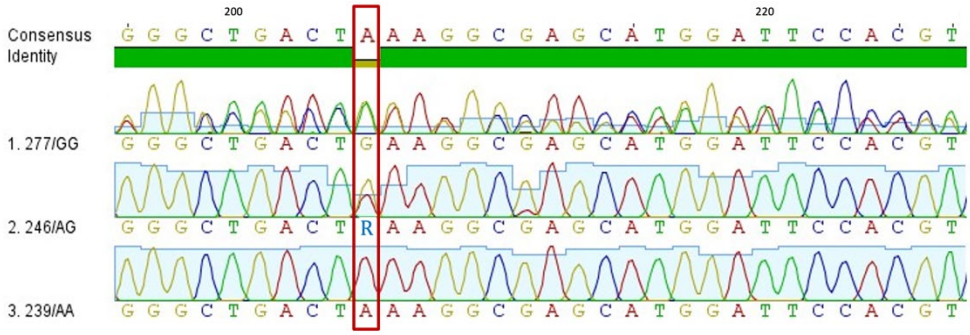

Fig. 3. The electropherograms of the *SOD1* gene from the exon 2 region hosting the *c.118G>A* mutation associated with CDM.

Legend: The red rectangle highlights the position of the SNP (Single Nucleotide Polymorphism). Sample 1. 277/GG belongs to a dog with the homozygous genotype G/G; Sample 2. 246/AG belongs to a dog with the heterozygous genotype A/G. Sample 3. 239/AA belongs to a dog with the homozygous genotype A/A.

6. Case studies

Five typologies of case studies were presented as part of this chapter in order to highlight the importance of genetic testing for CDM in a clinical setting. The five types of cases selected for presentation were: homozygous G/G case without clinical signs tested for screening purposes, homozygous G/G case with clinical signs tested in order to aid the differential diagnosis, heterozygous A/G case tested in order to aid the differential diagnosis, homozygous A/A case with clinical signs and homozygous A/A case with clinical signs with and possibly incomplete penetrance homozygous genotype.

III. Final conclusions and recommendations

The aim of this research was to conduct a comprehensive bibliographic study of neurologic diseases with known mutations in dogs and characterize them based on the level of documentation of the mutations. Additionally, the study aimed to perform a retrospective analysis to identify genetically diagnosed diseases in necropsied dogs, investigate the molecular technique used in the diagnosis of canine degenerative myelopathy, and estimate the prevalence of the mutation associated with canine degenerative myelopathy in purebred dogs in Romania.

Given the prevalence of autosomal recessive neurologic diseases, there is a need

for genetic counseling and breeding programs to reduce the incidence of these disorders in predisposed breeds like Golden Retrievers and Belgian Shepherds.

The extensive documentation of canine degenerative myelopathy highlights the importance of continued research and documentation efforts for better understanding and managing hereditary neurological disorders across various breeds.

The validation of the PCR-RFLP technique as specific, rapid, and effective suggests its potential for widespread use in genetic diagnostics, particularly for detecting canine degenerative myelopathy. The confirmation of PCR-RFLP results through Sanger sequencing reinforces the reliability of this diagnostic method, supporting its adoption in routine genetic testing for this disorder.

The detection of the *SOD1:c.118G>A* mutation in the Romanian Mioritic Shepherd for the first time, highlights the need for continued genetic surveillance and research in under-studied breeds to ensure comprehensive disease prevention strategies.

Recommendations

Knowledge of the genotype of a young animal with a known mutation can be beneficial for future clinical decisions. Considering that canine degenerative myelopathy (CDM) is an autosomal recessive disorder with incomplete penetrance, genetic testing is recommended for genetic prophylaxis to avoid the occurrence of dogs homozygous for the mutant allele *SOD1:c.118G>A*, as they will have a very high risk of developing CDM. Genetic testing is important for improving breeding programs in breeds known to be predisposed to canine degenerative myelopathy. It is worth noting that in the case of the Wire Fox Terrier breed, genetic testing for CDM has no value for diagnosis or selection for breeding, given the presence of the mutant allele in healthy individuals.

Genetic testing also has utility in the differential diagnosis of CDM and similar neurological disorders, such as degenerative lumbosacral syndrome, spinal cord neoplasms, intervertebral disc disease, cranial cruciate ligament rupture, and hip dysplasia.

Genetic testing can play a vital role in the efficient management and ultimately the eradication of inherited diseases. Recessive diseases pose a particular challenge for dog breeders, as parents are often asymptomatic carriers in the population, identified only retrospectively, usually after already producing affected offspring or, in some cases, after one parent has been diagnosed with the disease. This challenge is even more complicated when dealing with late-onset disorders, where affected dogs may be bred unknowingly with no knowledge of having a genetic condition. This issue is present both in dominant and recessive diseases.

Considering that most mutations have low frequencies, it can be difficult for any veterinarian to identify and have expertise in diagnosing a wide range of hereditary disorders. This underscores the importance of ongoing medical education and the presence of veterinary specialists with specific expertise in genetic counseling. Furthermore, emphasis is placed on the importance of comprehensive screening technologies.

IV. Originality and innovative contributions of the thesis

The section presenting the current state of knowledge related to this work aimed to present the molecular genetic techniques used in diagnosing Mendelian inherited disorders in dogs and performing an original classification study of 112 pathological phenotypes with known causal mutations of the canine nervous system, by adapting the ABC classification system used in human medicine. The study revealed that the majority of these diseases have an autosomal recessive mode of transmission, and dog breeds more predisposed to developing these conditions are Golden Retrievers (6 documented phenotypes) and Belgian Shepherds (5 documented phenotypes). Additionally, the most common types of mutations for hereditary neurological disorders in dogs are missense mutations (n = 46, 41%) and small deletions (n = 21, 19%). The original evaluation in the ABC system, presented for the first time in this work, revealed that 16 of the mutations were classified as pathogenic mutations, while the remaining 96 were classified as likely pathogenic mutations. To our knowledge, this is the first classification of this kind made for mutations associated with hereditary nervous system disorders in dogs. Moreover, the correlation of bibliographic data provides new research opportunities for genetic disorders of the nervous system in dog breeds where they have not yet been documented.

The testing of molecular genetic techniques applied in the diagnosis of degenerative myelopathy aimed to establish and validate an easily applicable protocol. The results indicated that buccal swab samples collected via minimally invasive methods from dogs are an adequate source of DNA testing for canine degenerative myelopathy. Moreover, among the two tested incubation protocols, the protocol adapted according to Holder et al. (2014) proved to be more effective due to the clearer amplicons, visualized after agarose gel electrophoresis migration. Furthermore, within this chapter, the first testing of dogs belonging to the Carpathian Shepherd breed was documented. The 19 tested individuals were homozygous with normal alleles (G/G), and the mutant allele A was not identified.

In the chapter dedicated to the molecular surveillance of the *SOD1:c.118G>A* mutation associated with canine degenerative myelopathy in Romania, 230 dogs belonging to 26 breeds were tested. To our knowledge, this study reports the first testing for canine degenerative myelopathy in dogs from the Romanian Shepherd, Caucasian Shepherd, and Romanian Mioritic Shepherd breeds. The *SOD1:c.118G>A*

mutation was identified for the first time in the Romanian Mioritic Shepherd breed, but not in the Romanian Shepherd and Caucasian Shepherd breeds. Additionally, the study was the first documentation and research of the surveillance of the mutation associated with canine degenerative myelopathy in Romania.

Sequencing of the six DNA samples obtained from dogs with all three genotype variants (G/G, A/G, and A/A) specific to MDC and analysis of the resulting electropherograms confirmed the results obtained through the PCR-RFLP technique.

The presented results can serve as a source of information to increase awareness of the presence of these types of conditions, the importance of their recognition, and the implementation of genetic prophylaxis measures to decrease their prevalence.

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