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DILATED CARDIOMYOPATHY IN THE DOMESTIC DOG (*CANIS LUPUS FAMILIARIS*) – *IN SILICO* ANALYSIS OF SELECTED GENES

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Abstract. Dilated cardiomyopathy (DCM) is a progressive loss of contractility of the heart muscle as the disease progresses. It causes a decrease in the heart's minute capacity, i.e. the volume of blood pumped by the heart into the blood vessels in one minute. DCM leads to congestive heart failure and sudden death. The aim of this study was to identify *in silico* genes within which mutations have occurred that may cause DCM in the domestic dog (*Canis lupus familiaris*), to identify dog breeds at risk, and to propose breed-specific diagnostic molecular tests. For bioinformatic analyses of sequences retrieved from GenBank (NC_006587.3 – *FGGY*, NC_006583.3 – *DCC* and CM023383.1 – *PDE3B*) and from scientific publications (*PDK4* – from patent publication number US 2011/0307965 A1 and *STRN* – Meurs et al. 2010), the following programs were used: Primer3 v. 0.4.0, NEBcutter v. 2.0 and BLAST. Based on literature data, domestic dog breeds such as Doberman Pinscher, Boxer, Portuguese Water Dog, Newfoundland, Irish Wolfhound and Great Dane were found to be among the breeds with the highest risk of DCM. In order to identify relevant mutations in the genes studied (*FGGY*, *DCC*, *PDE3B*, *PDK4* and *STRN*) that may cause the occurrence of dilated cardiomyopathy, the use of specific restriction enzymes has been proposed in molecular diagnostic tests: *Bmi*I for mutations in the *PDK4* gene and *Tth111*I for SNPs in the *FGGY* gene (Doberman Pinscher) and *Taq*I for SNPs in the *DCC* gene and *Hinf*I for SNPs in the *PDE3B* gene (Irish Wolfhound). This work, may serve as a prelude to analysis for targeted genetic testing to enable correct diagnosis of DCM in asymptomatic dogs.

Key words: domestic dog, dilated cardiomyopathy, DCM, left ventricular hypertrophy, PDK4.

INTRODUCTION

Dilated cardiomyopathy (DCM) belongs to the family of myocardial, endocardial or epicardial diseases. The diagnostic features are wall stretching and enlargement of the left ventricle (without an increase in cardiomyocytes), resulting in a loss of ability to make effective systole. This results in a backlog of blood in the cavities of the heart, which, as the disease progresses, leads to congestive failure of this organ and sudden death of the affected individual (Tidholm and Jönsson 2005; Sapierzyński 2013).

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DCM can be caused by many breed-specific factors. These include genetic (e.g. mutations) or environmental conditions such as taurine or carnitine deficiency, metabolic disorders such as diabetes mellitus or hypothyroidism, and immunologic conditions. It can also be a consequence of medications or diseases such as hypertension, heart valve defects or tachycardia (Tidholm and Jönsson 2005; Sapierzyński 2013; McNally and Mestroni 2018).

Three phases of progression are observed in dogs: asymptomatic, preclinical, and clinical. The full spectrum of symptoms is not apparent until the third phase of the disease and includes cough, dyspnea, apathy, syncope, and weight loss. The asymptomatic phase can last for years depending on the breed while the animal appears apparently healthy (Simpson et al. 2015a, b). In the preclinical phase, no clinical changes are observed, only electrical changes detected by electrocardiogram and/or morphological changes tested by echocardiogram (Simpson et al. 2015a, b).

Dogs at highest risk include large and giant breed dogs with a closed gene pool representing an isolated population with a "bottleneck" at the base of the breed (Mausberg et al. 2011). These include the Doberman Pinscher, Boxer, Irish Wolfhound, Portuguese Water Dog, the Great Dane, and Newfoundland. The course and diagnostic features of the disease are specific to each breed (Stabej et al. 2005).

The aim of this in silico study was to identify mutations within genes that DCM in selected domestic dog breeds, to identify dog breeds at risk and to propose breed-specific molecular diagnostic tests.

MATERIALS AND METHODS

Sequences used for *in silico* testing

In order to design individual diagnostic tests in three dog breeds such as the Doberman Pinscher, Irish Wolfhound and Boxer, the relevant sequences of selected genes, within which causal mutations have been reported in the literature (Meurs et al. 2010; Mausberg et al. 2011; Meurs 2011; Philipp et al. 2012; Simpson et al. 2016). Due to the unavailability of mutant sequences in GenBank, they had to be created in silico. In the present study, we focused on a 16-nucleotide deletion in the *PDK4* (Pyruvate Dehydrogenase Kinase 4) gene (Meurs 2011) and a SNP in the *FGGY* (Carbohydrate Kinase Domain Containing) gene in a Doberman Pinscher breed (Mausberg et al. 2011), a mutation within *STRN* (Striatin) in a Boxer (Meurs et al. 2010), or selected SNPs in the *DCC* (Netrin receptor DCC) (Simpson et al. 2016) and *PDE3B* (Phosphodiesterase 3B) gene in an Irish Wolfhound (Simpson et al. 2016).

Sequences for in silico analysis in Doberman Pinscher dogs

PDK4

In the mutation analysis (16 ntdel) of the *PDK4* gene, the correct sequence was obtained from patent publication number US 2011/0307965 A1 (Meurs 2011).

FGGY

For SNP analysis (CFA5:g.53,941,386T>C), the nucleotide sequences of the *FGGY* gene of domestic dog chromosome 5 were used, numbered NC_006587.3 (model sequence for the *Canis lupus familiaris* species of the Boxer dog), which are deposited in the GenBank database (https://www.ncbi.nlm.nih.gov/genbank/).

Sequence for *in silico* analyses in Boxer dogs

STRN

For mutation analysis (8 ntdel) within the *STRN* gene in the Boxer, a short fragment of the normal sequence was obtained from Meurs et al. (2010).

Sequences for *in silico* analysis in Irish Wolfhound dogs

DCC

For SNP analysis within the *DCC* gene (CFA1: rs.21953123C>T) in Irish Wolfhounds, a fragment of the domestic dog chromosome 1 sequence number NC_006583.3 was retrieved from the GenBank database (https://www.ncbi.nlm.nih.gov/genbank/).

PDE3B

For mutation analysis within the *PDE3B* gene (CFA21: g.40670543G>A) in Irish Wolfhounds, the corresponding sequence with reference number CM023383.1 for the SNP in chromosome 21 of the domestic dog was downloaded from the GenBank database (https://www.ncbi.nlm.nih.gov/genbank/).

METHODS

BLAST program (https://blast.ncbi.nlm.nih.gov/Blast.cgi) was used for *in silico* analyses to compare the similarity of wild-type and mutant sequences of the genes studied (Table 1).

Using the program Primer3 v. 0.4.0 software (http://bioinfo.ut.ee/primer3-0.4.0/) (Koressaar and Remm 2007; Untergasser 2012), suitable primers were designed and selected for use in the polymerase chain reaction to amplify the appropriate specific fragment of the genes under study.

The next step was to find a suitable restriction site and identify an appropriate restriction enzyme so that the wild-type and mutant sequence could be distinguished after cutting with a restrictase. The appropriate restriction enzyme should cut the amplified fragment at only one site. For this purpose, the NEBcutter v. 2.0 program (Vincze et al. 2003) was used (http://nc2.neb.com/NEBcutter2/). After selection of the optimal restrictase that cuts only one sequence (wild-type or mutated), using the NEBcutter v. 2.0 program (Vincze et al. 2003), visualization of the tested fragments in the specific agarose gel with indication of the mass standard appropriate to the length of the obtained restriction fragments was proposed.

RESULTS

Comparison of sequences in the BLAST program

The wild-type and mutant sequences of the studied genes were compared with each other using the BLAST program. The results are shown in Table 1. For the *STRN* gene, the available sequence fragment was too short, making it impossible to compare it or match a longer sequence to the known fragment, since no significant similarity was found in the database. Designed primer pairs for each of the selected genes are presented in Table 2.

Table 1. Comparison of wild-type and mutant sequences of tested genes in selected domestic dog breeds

Tested gene	Breed of domestic dog	Concordance [%]	Cause of difference
PDK4	Doberman Pinscher	95	Deletion of 16 nt in the intron folding site between exons 10 and 11 in the mutant gene variant
FGGY	Doberman Pinscher	99	Single nucleotide substitution at the CFA5:g.53941386T>C site
STRN	Boxer	–	–
DCC	Irish Wolfhound	99	Single nucleotide substitution at the CFA1:rs.21953123C>T site
PDE3B	Irish Wolfhound	99	Single nucleotide substitution at the CFA21g.40670543G>A site

Table 2. *In silico* designed primer pairs for a detection of mutations in selected genes associated with dilated cardiomyopathy in domestic dog

Tested gene	Forward primer (left)	Hybridization temperature of forward primers	Reverse primer (right)	Hybridization temperature of reverse primers	Amount of GC pairs
PDK4	atcagcagcagggaggata	59,80°C	aagccctaatcaggctcct	60,06°C	50%
FGGY	agtggatgaccacacagcc	59,58°C	aaacttcctggggatgctct	60,07°C	55% (f) 50% (r)
STRN	–	–	–	–	–
DCC	aggagacagtggctggcatt	62,18°C	agcatcctttgagaccgttg	60,25°C	55% (f) 50% (r)
PDE3B	gtgggcctcatccaatca	60,43°C	ccggtctcatgtgtcgtcat	62,00°C	55,56% (f) 55% (r)

In silico designed molecular test for detecting the 16bp deletion in the PDK4 gene in the Doberman Pinscher breed

In order to design primers using Primer3 v. 0.4.0 (Koressaar and Remm 2007; Untergasser et al. 2012), sequences whose lengths were 385 bp (wt) and 369 bp were used. Table 2 presents *in silico* designed primers.

Next using NEBcutter v. 2.0 software (Vincze et al. 2003), a restriction enzyme – *Bmr*I was selected to distinguish between mutant and wild-type sequence. This enzyme cuts the wild-type sequence only once. Because of the deletion the sequence "ACTGGG" is not present in the amplified fragment of the sequence derived from the mutant gene. In the next step it was recommended to visualize the resulting restriction products after cutting with the above enzyme in a 2% agarose gel in the presence of the Lambda marker – *Hind*III Digest. For the wild-type sequence – 215 bp and 170 bp were obtained and for the mutant sequence – 369 bp were obtained as it is not cut by *Bmr*I.

The above-described procedure is an *in silico* designed molecular diagnostic test for detecting carriers of the 16 ntdel in the *PDK4* gene (associated with dilated cardiomyopathy) in Doberman Pinscher dogs. Figure 1 shows a hypothetical view that, depending on the genotype of the individual for the *PDK4* (16 ntdel) mutation, can be obtained in a 2% agarose gel.

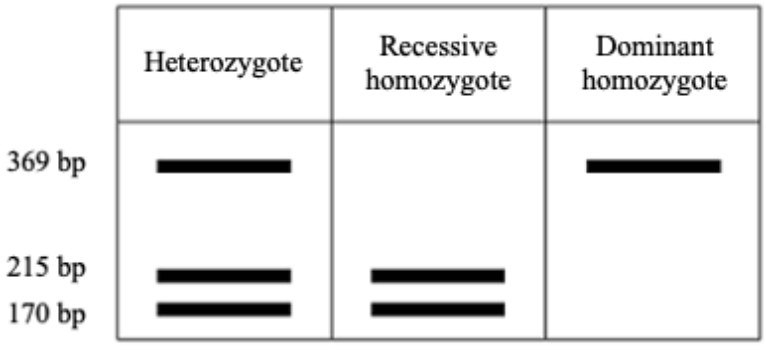

Fig. 1. Scheme representing distribution of DNA fragments in a 2% agarose gel according to the genotype of an individual in terms of mutation (16 ntdel) within the *PDK4* gene after cutting with *Bmr*I restriction enzyme (*in sillico* analysis)

***In silico* designed molecular test for detecting the (CFA5:g.53,941,386T>C) SNP in the *DCC* gene in the Doberman Pinscher breed**

Primers designed to amplify the selected fragment of the *FGGY* gene are presented in Table 2.

Using the NEBcutter v. 2.0 program (http://nc2.neb.com/NEBcutter2/) (Vincze et al. 2003), a suitable restriction enzyme *Tth111*I, was selected to identify mutations in the CFA5:g.53,941,386T>C sequence. It recognizes and cuts only the mutant sequence. For the wild-type mutation after cutting with the restriction enzyme *Tth111*I, the length of the visualized product in a 2% agarose gel is the same as the control (403 base pairs). In contrast, the mutant length is 200 bp and 203 bp. However, capturing the 3bp difference between the obtained restriction fragments was not possible in agarose gel. Therefore, to show the difference between the fragments, the mutant sequence was shortened to 230 nucleotides. PCR product was cut with restriction enzymes into two DNA fragments 167 bp and 63 bp long. The restriction enzyme that is *Tth111*I can be used to perform a molecular diagnostic test for the presence of a SNP in chromosome 5 (CFA5:g.53,941,386T>C) in a Doberman Pinscher dog.

Figure 2 shows a hypothetical separation of the DNA fragments in a 2% agarose HR gel after applying above-described *in-silico* designed molecular test.

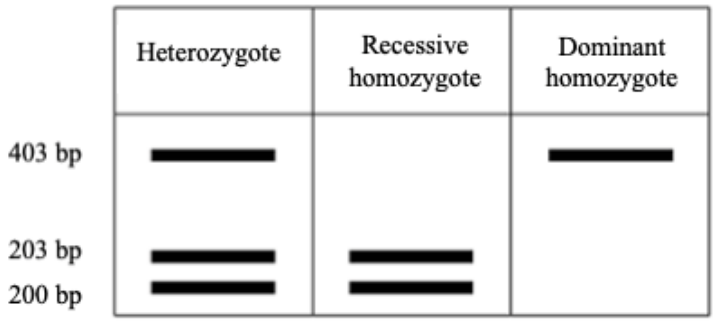

Fig. 2. Scheme of the distribution of fragments in a 2% agarose gel according to the genotype of an individual in terms of SNP mutation within the *FGGY* gene after cutting with *Tth111*I restriction enzyme (203bp and 200bp will be shown as a single strand)

In silico designed molecular test for detecting the (CFA5:g.53,941,386T>C) SNP in the *STRN* gene in the Boxer breed

Due to the fact that the entire sequence of the *STRN* gene is not available, the unmutated sequence of the *STRN* gene was found in the scientific article by Meurs et al. (2010). The available fragment was too short (47 bp in the wild-type sequence and 39 bp in the mutated sequence), which prevented the selection of suitable primers and would have prevented the amplification. Next, using the NEBcutter v. 2.0 program (http://nc2.neb.com/NEBcutter2/) (Vincze et al. 2003), attempts were made to select suitable restriction enzymes. However, none of them matched the basic assumptions (one restrictase can cut only one sequence wild-type or mutated), since both sequences were cut by the same enzymes. Therefore, it was not possible to select a suitable molecular assay having only these data.

In silico designed molecular test for detecting the (CFA1:C>T) SNP in the *DCC* gene in the Irish Wolfhound breed

In silico designed primers for the targeted *DCC* gene sequence are shown in Table 2.

Using the NEBcutter v. 2.0 program a *Taq*I restriction was selected to identify mutations in the CFA1:C>T sequence. This restriction enzyme recognises the following sequence: .

```
5'... T^C G A ... 3'
3'... A G C_^T ... 5'
```

A sequence of 59 nucleotides in length (so that the difference after cutting would be clearer) was used to visualize the PCR product in a 12% poliacryloamide gel in the presence of the

Lambda *Hind*III Digest marker, which was cut with the *Taq*I restriction enzyme. The wild-type sequence is 53 and 6 base pairs and the mutant sequence is the same as the original sequence, that is 59 bp.

The above-described procedure is an *in silico* molecular diagnostic test for detecting mutations within the CFA1:C>T SNP mutation in *DCC* gene.

Figure 3 shows a hypothetical view that, depending on the genotype of the individual for the *DCC* mutation, can be obtained in a 12% poliacryloamide gel.

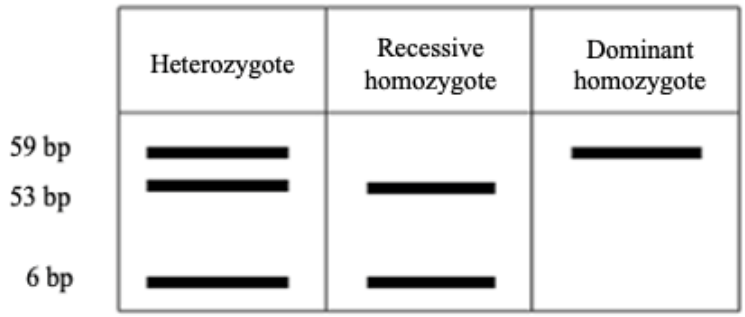

Fig. 3. Scheme of the distribution of fragments in a 12% poliacryloamide gel according to the genotype of an individual in terms of SNP mutation within the *DCC* gene after cutting with *Taq*I restriction enzyme

In silico designed molecular test for detecting the (CFA21:g.40670543G>A) SNP in the _PDE3B_ in the Irish Wolfhound breed

In silico designed primers for amplification of the targeted fragment of the *PDE3B* gene are presented in Table 2.

Using NEBcutter v. 2.0, the *Hinf*I restriction was selected to identify the CFA21:g.40670543G>A mutation. The *Hinf*I restriction enzyme cuts only the wild-type sequence. After cutting the wild-type sequence (144 bp) with the *Hinf*I restriction enzyme in a 2% agarose gel in the presence of the Lambda *Hind*III Digest marker, two fragments (132 bp and 12 bp) become visible.

The restriction enzyme *Hinf*I can be used to perform a molecular diagnostic test for the presence of a SNP in *PDE3B* gene in chromosome 21 (CFA21:g. 40670543G>A) in the Irish Wolfhound breed.

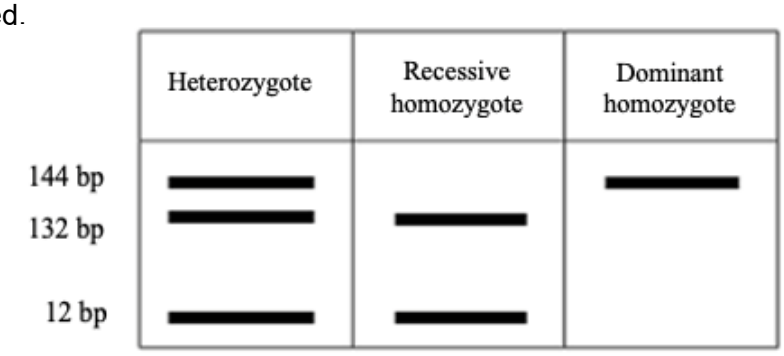

Fig. 4. Scheme of the distribution of fragments in a 2% agarose HR gel according to the genotype of an individual in terms of SNP mutation within the *PDE3B* gene after cutting with *Hinf*I restriction enzyme

Figure 4 shows a hypothetical view that, depending on the genotype of the individual for the *PDE3B* mutation, can be obtained in a 2% agarose HR gel.

DISCUSSION

Dilated cardiomyopathy disease occurs mainly in large and giant breed dogs but can occur in representatives of small breeds such as Cocker Spaniel. Supplementation with taurine and carnitine especially in the American population, has resulted in visible echocardiographic improvement (Dutton and Lopez-Alvarez 2018). Therefore, electrocardiographic and echocardiographic monitoring of the heart and testing the concentration of the amino acids' taurine and carnitine is very important, not only in at-risk large and giant breed dogs, but also, prophylactically, in smaller breed dogs. Particularly if changes are observed in any of the above tests.

On morphological examination (echocardiography), growth of the left ventricle is observed with thinning of the ventricular walls, which is directly associated with loss of systolic function. However, in 85% of all cases lesions involve all four cardiac cavities with left ventricular hypertrophy predominating (Tidholm and Jönsson 2005; Sapierzyński 2013). This may be related to the late diagnosis of the disease, at the time when it produces clinical symptoms and the prognosis of the individual is not successful, and treatment is no longer successful. Therefore, it is very important to find out the exact genesis of dilated cardiomyopathy in every breeds from the risk group, which unfortunately is not an easy task because all of the studies published in the literature and presented in this paper for the diagnosis of DCM, clearly indicate non-monogenic inheritance of this disease and not in all cases it is related to inadequate diet (taurine and carnitine deficiency). Diagnosis is often difficult due to the breed-specific features of the disease, e.g., in Newfoundland dogs, where the only early sign is a reduced left ventricular systolic fraction <22% in the absence of other cardiac abnormalities (Tidholm et al. 2000), while in Doberman Pinschers arrhythmias or ventricular premature complexes (VPCs) are characteristic (Bartoszuk-Bruzzone 2011). Additionally, differences occur not only within a breed, but also within a population. A perfect example is the controversy surrounding the impact of a mutation (16 ntdel) within the *PDK4* gene as a direct cause of dilated cardiomyopathy in Doberman Pinscher dogs. A study conducted on a population of American dogs of this breed showed a correlation between 16 ntdel in the *PDK4* gene and the occurrence of DCM in this breed (Meurs et al. 2012). Additionally, this gene variant has been shown to be absent in other dog breeds (Mausberg et al. 2011). However, studies in the European Doberman Pinscher population have not shown a significant association of the 16 ntdel mutation within the *PDK4* gene on the prevalence of DCM in Doberman Pinscher dogs (Owczarek-Lipska et al. 2012). It may be a direct result of the different habitat of these two populations and the difficult (often impossible or impractical, due to distance) mating of these animals.

Furthermore, this phenomenon supports the thesis of non-monogenic inheritance of dilated cardiomyopathy in this dog breed. This fact can be further confirmed by a genome-wide association study (GWAS) on the already cited European population (same research group). The localization of the *FGGY* gene mutation in chromosome 5, which contains the single nucleotide polymorphism SNP of the CFA5:g.53,941,386T>C and is characteristic for the occurrence of arrhythmia that accompanies the Doberman Pinscher breed in dilated cardiomyopathy (Mausberg et al. 2011).

In the present study, an attempt was made to design an appropriate molecular test for the rapid detection of mutations within the *STRN* gene in the Boxer breed, but due to the lack of available materials, e.g. a longer sequence, it turned out impossible to find a suitable restriction enzyme at this stage. Therefore, further research is recommended, starting with the sequencing of the entire *STRN* gene and determining the exact location of the mutation site.

In the Irish Wolfhound, SNPs on chromosomes 1, 21 and 37 have a significant effect on the occurrence of dilated cardiomyopathy (especially when they occur together) (Simpson et al. 2016). The present study examined their occurrence and identified the genes in which they are

located and suggested diagnostic tests for rapid mutations detection. A molecular diagnostic test has not been developed for the SNP in chromosome 37 as no sequence of this gene is available either in the literature or in GenBank. Therefore, it is proposed to re-sequence the entire nucleotide sequence and find the location of the corresponding SNP.

The molecular diagnostic tests described in this article have a potential to enable rapid diagnosis. Next step is to test designed PCR-RFLP reactions empirically in wet lab. Further research in this direction are required and may help in the treatment and prevention of dilated cardiomyopathy.

CONCLUSIONS

One of the most important aspects of proper breeding and care of animals, especially among breeds at risk of diseases such as dilated cardiomyopathy, is education. In the case of DCM, it is important to sensitize breeders/owners of dogs to react quickly and monitor animals that are in a group/breed at risk for the disease. Early detection of the disease, especially at the asymptomatic stage when the animal appears apparently healthy, prolongs this period and increases its chances of survival. In addition, information on the genotypes of owned animals will enable dog breeders/owners to exclude diseased dogs from breeding, thus reducing the risk of DCM in offspring generation.

Rapid detection of dilated cardiomyopathy is possible by monitoring at-risk dogs with electrocardiography (ECG), especially 24-hour Holter monitoring, showing electrical changes in the heart, and echocardiography (ECHO), which can detect morphological changes (e.g. left ventricular dilatation). Usually, these methods facilitate the detection of DCM in the preclinical phase, when no external symptoms are yet present. Blood taurine testing should also be an important parameter. Further studies should be carried out so that reference ranges for these tests for each breed are established.

Considering the above literature reports and the results of *in silico* analyses performed in this study, which can serve as a prelude to further research and indicate that dilated cardiomyopathy in the domestic dog has both genetic and environmental background.

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KARDIOMIOPATIA ROZSTRZENIOWA U PSA DOMOWEGO – ANALIZA *IN SILICO* WYBRANYCH GENÓW

Streszczenie. Kardiomiopatia rozstrzeniowa (ang. *dilated cardiomyopathy*, DCM) polega na postępującej wraz z rozwojem choroby utracie kurczliwości mięśnia sercowego. Powoduje zmniejszenie pojemności minutowej serca, czyli objętości krwi, która tłoczona jest przez serce do naczyń krwionośnych w ciągu minuty. DCM prowadzi do zastoinowej niewydolności serca i nagłej śmierci. Celem badań była identyfikacja *in silico* genów, w obrębie których wystąpiły mutacje, które mogą być przyczyną występowania DCM u psa domowego (*Canis lupus familiaris*), a także określenie ras psów będących w grupie ryzyka oraz zaproponowanie odpowiednich dla danej rasy diagnostycznych testów molekularnych. Do analiz bioinformatycznych sekwencji, pobranych z GenBanku (NC_006587.3 – *FGGY*,

NC_006583.3 – *DCC* i CM023383.1 – *PDE3B*) i z publikacji naukowych (*PDK4* – z opisu patentowego US 2011/0307965 A1 oraz *STRN* – Meurs i in. 2010), wykorzystano następujące programy: Primer3 v. 0.4.0, NEBcutter v. 2.0 oraz BLAST. Na podstawie danych z literatury stwierdzono, że rasy psa domowego, takie jak doberman pinczer, bokser, portugalski pies wodny, nowofundland, wilczarz irlandzki i dog niemiecki, zaliczane są do ras o największym ryzyku wystąpienia DCM. W celu identyfikacji odpowiednich mutacji w badanych genach (*FGGY*, *DCC*, *PDE3B*, *PDK4* oraz *STRN*), które mogą być przyczyną występowania kardiomiopatii rozstrzeniowej, zaproponowano zastosowanie w molekularnych testach diagnostycznych specyficznych enzymów restrykcyjnych: *Bmi*I dla mutacji w genie *PDK4* i *Tth111*I dla SNP w genie *FGGY* (doberman pinczer) oraz *Taq*I dla SNP w genie *DCC* i *Hinf*I dla SNP w genie *PDE3B* (wilczarz irlandzki). Praca ta może stanowić wstęp do analiz pod kątem celowanych badań genetycznych, które umożliwią prawidłową diagnozę DCM u psów w fazie bezobjawowej.

Słowa kluczowe: pies domowy, kardiomiopatia rozstrzeniowa, DCM, przerost lewej komory serca, PDK4.