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PCR DETECTION OF AN EYE ANOMALY IN A FAMILY OF LONGHAIRED COLLIES

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ABSTRACT

Inherited eye diseases have been the subject of genetic research for many years. This paper focuses on the optimisation of the DNA test based on the polymerase chain reaction (PCR) for the detection of Collie Eye Anomaly (CEA) in dogs. A small family of four longhaired Collies (parents and their daughters) with a confirmed positive clinical ophthalmologic examination of CEA served as the source of affected animals. Both PCR reaction conditions examined were suitable for detecting canine *NHEJ1* gene mutation associated with CEA. One carrier was found in a small group of eleven randomly selected control healthy dogs. The PCR test confirmed the previous CEA-positive ophthalmological examination in Collies. The results indicated that all four family members of the examined longhaired Collies had a homozygous intronic deletion of 7799 bases in the canine *NHEJ1* gene. The affected female Collies may potentially transmit this CEA-associated mutation to their puppies.

Key words: Collie eye anomaly; dogs; hereditary eye diseases; PCR

INTRODUCTION

Hereditary eye diseases are becoming a serious problem in some breeds of dogs. In diagnosing these conditions, the first step is usually a clinical ophthalmological examination. However, in many cases with a genetic basis, specific genetic tests are also welcome to complement a comprehensive clinical examination. This is especially valuable in diseases with similar symptoms where molecular genetic methods help to refine the diagnosis. In addition, genetic tests are important in detecting asymptomatic carriers (heterozygotes) of recessive alleles in the case of autosomal recessive inheritance modes. When crossing with another carrier, heterozygotes may pass these alleles on to their offspring (Fig. 1). A congenital condition, Collie Eye Anomaly (CEA), was an example of that transfer in multiple dog breeds for many years [10, 12, 13]. However, there are doubts about the monogenic autosomal recessive inheritance of this disease at this time [3], based on research of Nova Scotia Duck Tolling Retrievers.

The CEA anomaly is not simple but complex in nature with the prevalent phenotype of choroidal hypoplasia in mild cases. More severe cases may also have optic nerve colobomas, retinal detachment and intraocular haemor-

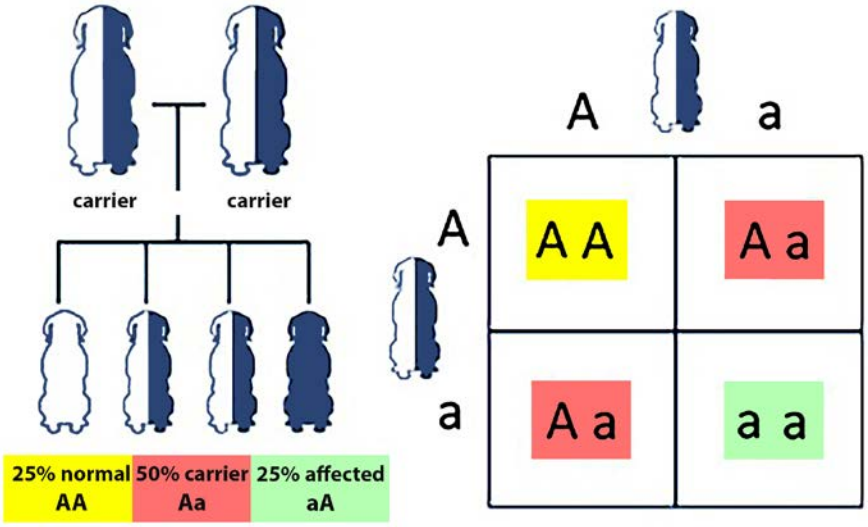


Fig. 1. Crossing of two asymptomatic carriers (Aa): 25 % of puppies are affected (aa)
Source: https://www.pawprintgenetics.com/blog/2018/11/13/who-test-autosomal-recessive-genetic-diseases/

rhage [7]. The current paper of Kucharczyk et al. [10] describes CEA as a congenital canine pleomorphic ocular disorder characterized by two main lesions: choroidal hypoplasia/chorioretinal dysplasia (CH/CRD) and papillary/peripapillary colobomata. The CH/CRD is bilaterally present, with varying degrees within the same individual and between affected dogs. As concerns colobomata, their size varies; the larger defects may be involved in post-natal retinal detachment. Similarly, Mizukami et al. [11] pointed out that CEA is a syndrome with variability in manifestation and severity of clinical and ophthalmological lesions. The most severely affected dogs may develop retinal detachment and intraocular bleeding leading to blindness [2, 6, 14]; however, this is usually with a low incidence.

CEA was first described in the Collie and Shetland sheepdogs and later in other breeds [1, 3, 11]. The eye examination is usually advisable at six weeks of age.

In 2007, Parker et al. [13] developed the scientific basis for DNA testing of CEA. The authors revealed that a large homozygous deletion of 7.8 kb within an intron 4 in the *NHEJ1* gene (non-homologous-end-joining factor 1; located on chromosome 37) was present in all CEA-affected dogs and absent in unaffected dogs of multiple breeds.

The aim of our study was to optimize the polymerase chain reaction (PCR) conditions for the detection of hereditary Collie Eye Anomaly (CEA) in groups of both the controlled clinically healthy dogs, and the affected animals. The affected dogs were members of a small family of longhaired collies with a confirmed positive clinical ophthalmologic examination of CEA.

MATERIALS AND METHODS

Sample collection

Blood and/or buccal (cheek) cells of dogs served as a source of DNA. The total number of dogs tested was 15. Of these 15 dogs, four came from the same family of longhaired Collies. The family consisted of mother (age 6.5 years), father (age 9.5 years), daughter 1 (age 4 years) and daughter 2 (age 11 months). In all of these Collies, CEA had been confirmed by ophthalmologic examination in previous years (as stated by the owner), but this finding was not supported by genetic verification. Eleven (11) ophthalmological healthy dogs of different breeds and ages (1 year to 14 years) served as phenotypically unaffected controls.

The whole blood samples were collected at the University Veterinary Hospital (Small Animal Clinics) into sterile EDTA tubes (0.5 M EDTA, pH 8.0) from seven dogs of the following breeds: Yorkshire terrier, Golden retriever, Rhodesian ridgeback, Bulldog, Bernese mountain dog, Bavarian painter and Hanoverian painter.

As for the buccal swabs of the dogs' mucosal cells, their owners mostly took the swabs. They used sterile cytology brushes according to the instructions of the Genomia laboratory (https://www.genomia.cz). These samples came from eight dogs of the following breeds: Labrador retriever (one dog), Jack Russell terrier (one dog), Border collie (one dog), Shetland sheepdog (one dog) and four above-mentioned longhaired Collies.

ETHICAL CONSIDERATIONS

All procedures involving animals followed the guidelines stated in the Guide for the Care and Use of Animals (Protocol No. 3323/16-221/3) approved by the State Veterinary and Food Administration of the Slovak Republic and by Ethics Commission of the University of Veterinary Medicine and Pharmacy in Košice, Slovakia.

Isolation of DNA

Genomic DNA was extracted using binding membrane-based systems (binding columns in microcentrifuge tubes) of two kits: Whole Blood Genomic DNA Fast Extraction kit (BioTeke, China) and ReliaPrepTM Blood gDNA Miniprep System (Promega, Madison, USA). Both kits were designed to extract DNA from whole blood, so the procedure for isolating DNA from buccal swabs was slightly modified. The main modifications included extending the lysis time (30 minutes instead of 10) and gentle thorough mixing of the cells before and after lysis (20 seconds instead of 10). The concentration and purity of DNA were determined at 260 and 280 nm using NanoPhotometer (Implen GmbH, Germany).

Primers and PCR reactions

Detection of CEA-associated deletion in the *NHEJ1* intron 4 was performed by PCR amplification with primers described in P a r k e r et al. [13]. Two sets of primers were used: *NHEJ1*-F17/*NHEJ1*-R17 to amplify within the deletion and *NHEJ1*-F20/*NHEJ1*-R23 to amplify across the deletion. The sequences of the primers were as follows:
NHEJ1-F17 5'-TCTCACAGGCAGAAAGCTCA-3'
NHEJ1-R17 5'-CCATTCATTCCTTTGCCAGT-3'and
NHEJ1-F20 5'-TGGGCTGGTGAACATTTGTA-3' /
NHEJ1-R23 5'-CCTTTTTGTTTGCCCTCAGA-3'.

Two final modifications of the PCR reaction were used, as indicated in Table 1.

Tab. 1. Final modifications of PCR conditions for CEA detection

Step	Reaction conditions 1		Reaction conditions 2	
	Temp [°C]	Time [s]	Temp [°C]	Time [s]
Initial denaturation	95	120	95	120
Denaturation	95	40	95	30
Annealing	50	50	55	30
Synthesis	72	90	72	60
Final synthesis	72	300	72	300

PCR reaction mixture (25 ml) consisted of 14.5 ml water, 5 ml buffer (5× Go TaqÒ Flexi Buffer, Promega), 2.5 ml $MgCl_2$ (25 mM), 0.5 ml dNTP (10 mM each), 0.625 ml of each primer (10 mM), 0.25 ml DNA polymerase (GoTaqÒG2Hot Start Polymerase, Promega) and 1 ml of genomic DNA.

Agarose gel electrophoresis

Agarose gel electrophoresis was performed to visualise the PCR results (amplicons) using 1.5 % agarose in 1×TAE buffer (1 h, 75 V, Gel Haus 300). Gels were stained by GelRed® (Biotinum, CA) and documented via UV light using the Digimage system (Major Science, USA).

RESULTS

The conditions of the PCR reaction 1 (Tab. 1) were shown to be suitable for amplification and detection of the resulting amplicon on a 1.5 % agarose gel. Using the first set of primers (*NHEJ1*-F17/*NHEJ1*-R17) that flank a sequence within a site of potential deletion, it is possible to determine whether the subject is healthy (normal, clear) (AA), i. e., no deletion is present in intron 4 of the *NHEJ1* gene (Fig. 2). Regarding the carrier status, the PCR reaction of the same individual with the second set of primers should be performed (Fig. 3, Fig. 4). This allows distin-

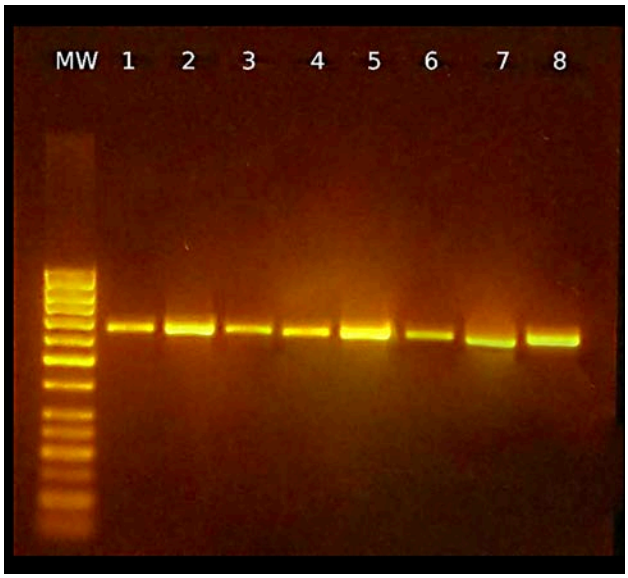


Fig. 2. Agarose gel with resulting PCR amplicons (636 bp) in healthy dogs
MW – Standard of molecular weight 50 bp; lanes 1, 2 – Labrador retriever; lanes 3, 4 – Jack Russell terrier; lanes 5, 6 – Yorkshire terrier; 7, 8 – Golden retriever

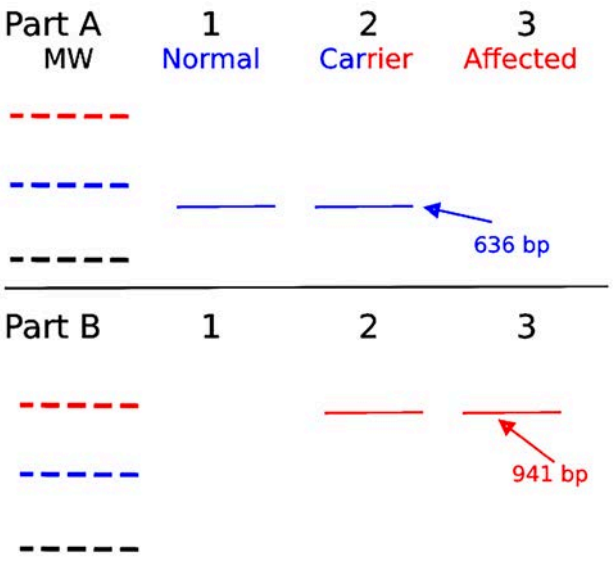


Fig. 3. Schematic representation of PCR results on agarose gel using two pairs of primers for each of the tested individuals 1, 2 and 3
Part A: the PCR results on agarose gel using the first set of primers (*NHEJ1*-F17/*NHEJ1*-R 17) for individuals 1, 2 and 3. **Part B:** the PCR results on the same agarose gel using the second pair of primers (*NHEJ1*-F20/*NHEJ1*-R23) in the same individuals. Considering simultaneously parts A and B of the same gel the individual 1 is normal (clear; healthy) (AA), individual 2 is a carrier (Aa) and individual 3 is genetically CEA-affected (aa)

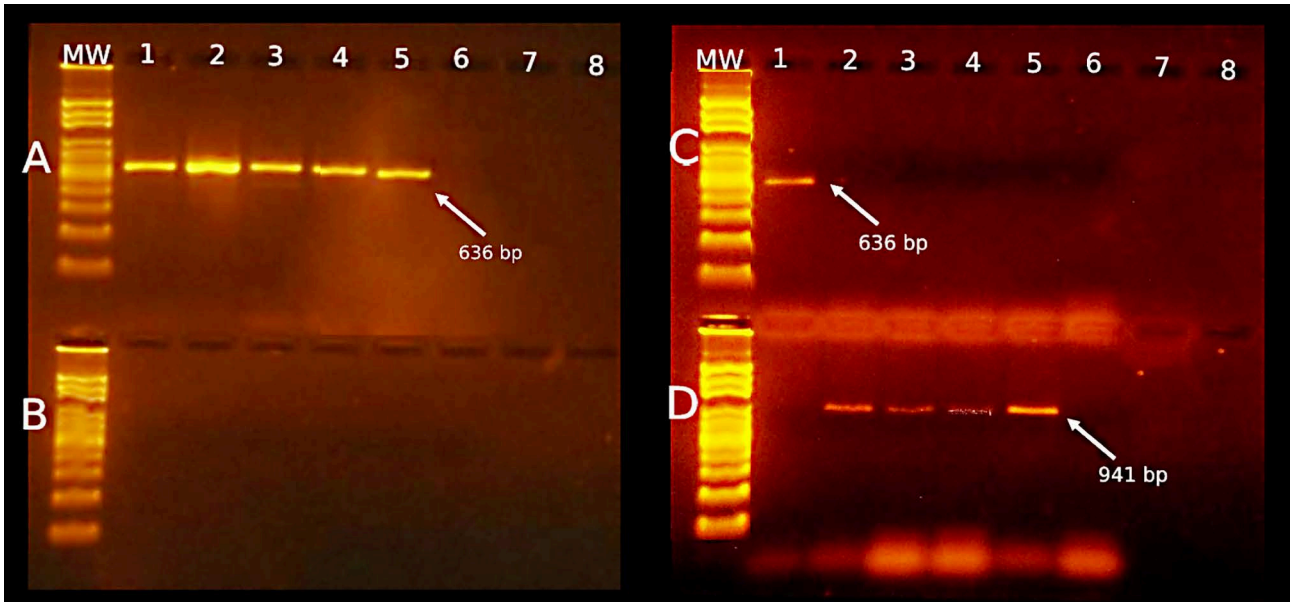


Fig. 4. PCR detection of normal (clear) and genetically CEA-affected dogs
Agarose gel 1 – normal (clear) dogs: MW – standard of molecular weight 100 bp; line 1 Rhodesian ridgeback; line 2 Bulldog; line 3 Bernese mountain dog; line 4 Bavarian painter; line 5 Hanoverian painter; line 6 negative control (water instead of DNA). Agarose gel 2 – normal (clear) (line 1) and CEA-affected dogs (lines 2–5): line 1 Border collie; line 2 Longhaired collie – father; line 3 Longhaired collie – mother; line 4 Longhaired collie – daughter one; line 5 Longhaired collie – daughter two; line 6 – negative control (water instead of DNA)

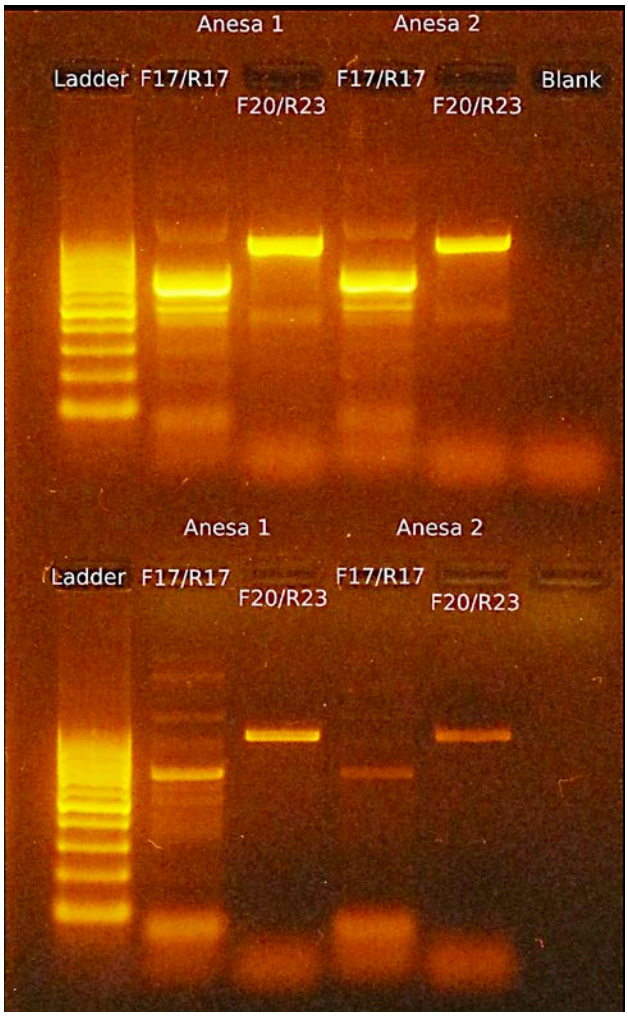


Fig. 5. PCR detection of amplicons in a Shetland sheep dog carrier of CEA
Agarose gel: line 1 ladder 100 bp; lines 3–5 positive PCR reactions with both sets of primers (*NHEJ1*-F17/*NHEJ1*-R 17 and *NHEJ1*-F20/*NHEJ1*-R23) indicate a carrier dog Anesa (636 bp and 941 bp); line 6 – negative control (water instead of DNA). At the bottom of the picture is the PCR result with tenfold diluted DNA

guishing healthy individuals (dominant homozygotes AA) from phenotypically normal carriers of recessive allele (heterozygotes Aa) (Fig. 3; Fig. 4/agarose gel 1) as well as to detect genetically affected animals (aa) (Fig. 4/agarose gel 2). Despite slightly less visible PCR amplicons, the second PCR reaction conditions tested (Reaction conditions 2, Table 1) with increased annealing temperature (from 50 °C to 55 °C) and shortening times of denaturation, annealing and synthesis showed to be sufficient for the detection of resulting fragments on an agarose gel. The results are on Fig. 4 (agarose gel 2), where is visible one amplicon of healthy Border collie and four amplicons of longhaired Collie family members (mother, father and two daughters) that were affected by CEA disease.

The result of the PCR testing of a CEA carrier dog (Shetland sheepdog) is in Fig. 5.

DISCUSSION

Although the genetic test for CEA is commercially available nowadays (e. g., Laboklin s. r. o.), we attempted to test the PCR reaction conditions for CEA detection using two sets of *NHEJ1* primers described in the scientific literature. Both PCR reaction conditions tested showed to be suitable for the visualisation of specific PCR amplicons (indicated the presence or absence of *NHEJ1* gene mutation – deletion) on an agarose gel (Table 1, Fig. 2, Fig. 4, Fig. 5). Similar results were obtained by D o s t á l et al. [6] using the same primers as in our study, but more extreme temperature of denaturation (98 °C), very short times and different chemicals. The authors did not isolate DNA but used a direct PCR amplification using the 20-times diluted unclotted blood samples. On the contrary, in our study, we used DNA isolated from the blood and buccal cells; we slightly modified mainly isolation method from buccal cells that are a good and non-invasive source of DNA. In addition, saliva specimens can serve for DNA analysis using the PCR, especially the real-time PCR with a melting curve analysis, as shown by C h a n g et al. [9] and confirmed by M i z u k a m i et al. [11] in the case study in Hokkaido dogs affected by CEA.

As indicated by G o u g h et al. [7], Collie Eye Anomaly is best diagnosed at 6–7 weeks. Similarly, C r i s p i n and M e l l e r s h [5] emphasised that CEA is a congenital condition, which can be diagnosed as soon as eye examination is possible (at five to six weeks of age), and it is diagnosed clinically with greatest accuracy in such young dogs. On the contrary, a genetically affected dog may be clinically unaffected at the time it has an eye examination, especially if it is very young [8].

According to G r o s å s et al. [8] who examined choroidal hypoplasia in 103 Norwegian Border Collie puppies in the age from 5 to 8 weeks, choroidal hypoplasia/chorioretinal dysplasia (CH/CRD) is consistently visible on ophtalmological examination only until the age of 10 weeks. Later development of the tapetal area may mask the lesions resulting in so-called “go-normal” phenomena [2]. For this reason, the authors recommend the clinical eye examination prior to the age of 10 weeks [2, 8]. In gen-

eral, it is recommended to perform a preventive clinical examination of the eyes before placing the dog in the breed [15]. To eliminate CEA from a breed, complex examination such as litter screening combined with DNA testing is proposed as the best approach [3, 5].

There is currently much debate about the type of inheritance in relation to CEA. The polygenic type of inheritance or single-gene coded (autosomal recessive) inheritance with incomplete penetrance was supposed for this disorder [12]. Moreover, ophthalmic phenotypes such as choroidal hypoplasia and coloboma are currently thought to be inherited separately. The collection of optic nerve head (ONH) coloboma affected Nova Scotia Duck Tolling Retrievers showed lack of concordance of the *NHEJ1* intronic deletion with ONH coloboma. Due to the complex genetic aetiology of ONH coloboma, the *NHEJ1* intronic deletion test results should be carefully considered when making breeding decisions [3].

Dog genetics is an interesting and very useful area of research. Some canine diseases are clinically similar to human diseases with the same gene involved in dog and humans [4]. Mainly, for this reason, a biological resource centre of canine samples Cani-DNA CRB (http://dog-genetics.genouest.org) now serves as a biobank of blood and tissue samples from affected and unaffected dogs for genetic studies.

CONCLUSIONS

Both PCR reaction conditions tested were suitable for detecting canine *NHEJ1* gene mutation associated with CEA using two different sets of primers in each individual. One carrier was observed in a small group of eleven randomly selected healthy dogs. Moreover, the PCR test confirmed the previous CEA-positive ophthalmological examination in Collie breeding. This test indicated that all four family members (mother, father and two daughters) of the examined longhaired Collies were genetically affected by homozygous intronic deletion of 7799 bases in the canine *NHEJ1* gene. The two young female Collies (daughters) may potentially transmit this CEA-associated mutation to the next generation of offspring. For this reason, it is advisable to remove affected dogs from a breeding programme in most cases.

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