FACULTY OF HEALTH AND MEDICAL SCIENCES
UNIVERSITY OF COPENHAGEN

Master's thesis

Marie Jönsson zlm536
Marie Annie Söderlund rks773

Investigating the mutation in the *NHEJ1* gene in the Danish Rough Collie

Academic advisor: Merete Fredholm, Professor

Section for Animal Genetics, Bioinformatics and Breeding
Institute for Clinical and Veterinary Sciences
Faculty of Health and Medical Sciences
University of Copenhagen

Submitted: 28/02/2014

Abstract

Collie eye anomaly (CEA) is a widespread congenital hereditary eye disorder in Collies all over the world, and it has been suggested that most Collies are carriers of CEA. Chorioretinal dysplasia (CRD), the primary phenotype of CEA, is a congenital and non-progressive defect, diagnosed by ophthalmoscopic examination of puppies at the age of 5-10 weeks. CRD affected dogs have been found to share a homozygous intronic deletion of 7.8 kbp in the *NHEJ1* gene. Today a genetic test is available for CRD. This study investigates the compliance between genetic and clinical diagnosis of CRD, and tries to find out if any genetically unaffected Rough Collies can be identified.

A literature review was performed in order to account for the different aspects of CEA and to highlight previous research of relevance. Additionally, a genetic case-control study was carried out, in which the presence of the mutation in the *NHEJ1* gene was compared to the clinical diagnosis of CRD in 45 Rough Collies. All the dogs were clinically diagnosed before the age of 10 weeks. Buccal swabs were collected from the dogs, the DNA was isolated and then genotyped using conventional polymerase chain reaction (PCR).

The results of this study show that the compliance between the clinical and genetic diagnosis is poor. Out of the 45 dogs, 44 were homozygous for the mutation in the *NHEJ1* gene, and one dog was heterozygous, i.e. a genetic carrier or CRD. Thereby, none of the Rough Collies in our study were genetically unaffected by the mutation. Conclusively, the results obtained in this study suggest that the available genetic test for CRD cannot be relied upon.

Resumé

Collie eye anomaly (CEA) är en utbredd, medfödd och ärftlig ögonsjukdom bland collier i hela världen, och det har insinuerats att de flesta collier är bärare av CEA. Chorioretinal dysplasia (CRD), den primära fenotypen av CEA, är en medfödd, icke-progressiv defekt, som diagnostiseras genom oftalmoskopisk undersökning av valpar, 5-10 veckor gamla. Hundar med CRD har bevisats dela en gemensam homozygot intron deletion på 7,8 kb i *NHEJ1*-genen och idag finns ett genetiskt test för CRD tillgängligt. Denna studie undersöker överensstämmelsen mellan genetisk och klinisk diagnos av CRD, och försöker ta reda på om det går att identifiera långhåriga collier som är genetiskt fria från CRD.

En litteraturstudie utfördes för att redovisa bakgrunden till CEA och för att framhäva tidigare forskning av relevans. Därutöver gjordes en fall-kontrollstudie, vari förekomsten av mutationen i *NHEJ1* genen sammanliknades med den kliniska diagnosen av CRD hos 45 långhåriga collier. Alla hundarna var kliniskt diagnostiserade innan 10 veckors ålder. Buckala svabbprover från hundarna samlades in, varefter DNA isolerades och genotypades med hjälp av konventionell polymerase chain reaction (PCR).

Resultaten från denna studie visar att överrensstämmelsen mellan den kliniska och genetiska diagnosen är svag. Av 45 hundar var 44 homozygota för mutationen i *NHEJ1* genen, och en hund var heterozygot, alltså en genetisk bärare av CRD. Därav var ingen av de långhåriga collierna i denna studie genetiskt fri från mutationen. Sammanfattningsvis tyder resultaten från denna studie på att det genetiska test som finns tillgängligt för CRD inte går att förlita sig på.

Preface

This master´s thesis is the final part of our veterinary degree, and is written in the field of veterinary medicine at the Section for Animal Genetics, Bioinformatics and Breeding, Institute for Clinical and Veterinary Sciences, Faculty of Health and Medical Sciences, University of Copenhagen.

The intention of the study is to increase the knowledge of the genetic prevalence of CEA in Denmark, as well as investigate the compliance between the results of the genetic test for CRD and the clinical diagnosis. The target audience of this thesis is veterinarians, veterinary students, dog breeders and dog owners with an interest for CEA and the genetics of the disease.

Our acknowledgements go to the Danish Collie Club and the Danish Kennel Club for contributing with funding. Thank you Tina Bahrt Neergaard Mahler and Christel Ammitzböll Halberg for providing us with guidance, expertise and helpfulness during laboratory sessions. We would also like to thank veterinary Ida Möller, ophthalmology specialist, at Evidensia Djursjukhuset Helsingsborg for the opportunity to participate during clinical examinations and her time answering our questions. Finally, very special thanks to our supervisor, professor Merete Fredholm, for the support, guidance and great commitment in the process of our master´s thesis.

28th of February 2014

Marie Annie Söderlund rks773

Marie Jönsson zlm536

Abbreviations

A: Adenine

BAC: Bacterial artificial chromosome.

Bp: Base pair

C: Cytosine

CEA: Collie eye anomaly

CFA37: Canine chromosome 37

CRD: Chorioretinal dysplasia

DNA: Deoxyribonucleic acid

G: Guanine

Kbp: Kilo base pair

$MgCl_2$: Magnesium Cloride

NHEJ1: Non-homologous end-joining factor 1

PCR: Polymerase chain reaction

SNP: Single nucleotide polymorphism

***Taq*:** *Thermus aquaticus*

T: Thymine

dNTP: Deoxynucleotide triphosphates

Glossary

7.5x Boxer sequence assembly: A high quality sequence assembly obtained from a female boxer. The sequence spans most of the dog's 2.4 billion bases in a sum total of 31.5 million sequence reads**.**

Allele: One of two or more alternative forms of a gene at the same site or locus in each of a pair of chromosomes, which determine alternative characters in inheritance.

Autosomal: Any chromosome other than the sex chromosomes.

Candidate gene: A DNA sequence in a chromosome region suspected of being involved in a particular disease of interest, whose protein product suggests that it could be the disease gene in question.

Contig: A set of overlapping clones.

Deletion: Loss of genetic material from a chromosome.

Deoxynucleotides: Components of DNA, containing the phosphate, sugar and organic base.

Dominant: Capable of expression when carried by only one of a pair of homologous chromosomes.

Exon: Regions of a primary RNA transcript in eukaryotic cells that are coding and are joined together when introns are spliced-out, to make the functional mRNA.

Flanking regions: Noncoding sequences on either side of the coding region of a gene that contain various regulatory sequences.

Gene pool: Total of all genes possessed by all members of the population which are capable of reproducing during their lifetime.

Genotype: The genetic makeup of a cell.

Haplotype: The group of alleles of linked genes contributed by either parent.

Heterozygous: Having different alleles at one locus.

Homozygous: Having the same alleles at one locus.

Identical by descent (IBD): Alleles in an individual or in two people that are known to be identical because they have both been inherited from a demonstrable common ancestor.

Intron: Untranslated, intervening sequences that are interspersed between coding sequences of a particular gene.

Linkage disequilibrium (LD): A statistical association between particular alleles at separate but linked loci, normally the result of a particular ancestral haplotype being common in the population studied.

Locus: The specific site of a gene on a chromosome.

Major gene: A gene that is necessary and sufficient by itself to cause a condition.

Modifier gene: Genes that have small quantitative effects on the level of expression of another gene.

Mutation: A nucleotide change, including base substitutions, insertions or deletions in DNA, or RNA in the case of some viruses, that gives rise to the mutant phenotype.

Nucleotides: Any of a group of compounds obtained by hydrolysis of nucleic acids.

Oligonucleotides: A polymer made up of a few to a hundred or more nucleotides.

Penetrance: The frequency with which a genotype manifests itself in a given phenotype.

Phenotype: The observable characteristics of a cell or organism, including the result of any test that is not a direct test of the genotype.

Polygene: A group of nonallelic genes that interact to influence the same character with additive effect.

Prevalence: The total number of cases of a specific disease in existence in a given population at a certain time.

Primer: Oligonucleotide which is hydrogenbonded to the template strand of DNA; required for the replication of DNA by polymerase.

Polymerase: An enzyme that catalyses polymerization, particularly of nucleic acids.

Recessive: A character is recessive is it is manifest only in the homozygote.

Contents

Abstract

Resumé

Preface

Abbreviations

Glossary

Introduction 1

Background 1

Aim 2

Delimitation 3

Method 3

Relevant Anatomy of the Eye 4

Sclera 4

Choroid 4

Retina 5

Optic nerve 5

Clinical Pathology of CEA 6

Clinical Features 6

Chorioretinal Dysplasia 6

Coloboma 7

Secondary Changes 8

Embryology and Pathology 10

Clinical Diagnosis 10

Genetics 12

Genetic Mapping of CRD 14

Genotyping using conventional PCR 16

Breeding Recommendations 18

Materials and Method 19

Materials 19

Method 20

Isolation of total DNA 20

Polymerase Chain Reaction 20
Results 22
Discussion 24
Conclusion 27
Perspectives 27
References 29
Appendix 1 32
Appendix 2 34
Appendix 3 38
Appendix 4 41
Appendix 5 43

Introduction

Background

Collie eye anomaly (CEA) is a widespread congenital hereditary eye disorder in Collies all over the world, with a prevalence of 64% in the UK (Bedford 1982b), 31% in Finland (Leppänen & Saloniemi 1998), 41% in Norway (Bjerkås 1991) and 41% in the Netherlands (Stades & Barnett 1981). Due to the very high prevalence in some countries, it has been suggested that most Collies are carriers of CEA (Bedford 1982b).

Magrane, who discovered the ophthalmological defects in the Collie, first described the disorder in 1953. Since then, it has been found that various other breeds are affected by CEA. Because of this, the name of the disorder can be misleading, and the term "congenital posterior segment anomaly" has been suggested as an alternative term to CEA (Bedford 1998). Despite this, the name of the disorder remains unchanged.

Diagnostically, it is differentiated between chorioretinal dysplasia (CRD) and coloboma. CRD is the primary phenotype of CEA but has the least effect on vision. The abnormality affects both eyes but it appears most often asymmetrically (Bedford 1982b). CRD affects the retina and/or the choroid, impairing development and causing absence of pigment. The defect is congenital, non-progressive, and diagnosed by ophthalmoscopic examination of puppies at the age of 5-10 weeks (Barnett 1979). It is crucial that the diagnosis is assigned before the age of 10 weeks. This is due to the retina changing colour from blue to yellow/green and possibly "masking" the changes, making CRD difficult to detect (Bjerkås 1991). This phenomenon is called "go normal" (Bedford 1982b).

Coloboma is a much more severe abnormality of CEA. This abnormality causes pits in the optic disc or its surrounding region, which in turn affects vision. Different complications can be seen together with coloboma, including vascular changes, retinal detachment and blindness (Roberts *et al.* 1966).

CRD is generally recognized to be inherited as an autosomal recessive trait (Lowe *et al.* 2003). Some authors believe CRD to be inherited as a simple autosomal recessive trait (Donovan *et al*. 1969), although others have suggested that it more likely is inherited as a polygenic autosomal recessive trait (Wallin-Håkanson *et al.* 2000b).

The locus of CRD was mapped to the canine chromosome 37 (CFA37) (Lowe *et al.* 2003) and affected dogs have been found to share a homozygous intronic deletion of 7.8 kbp in the *NHEJ1* gene (Parker *et al.* 2007). This finding has led to a genetic test for CRD being available. Further investigations of the compliance between clinical and genetic diagnosis of CRD would be of interest. If the compliance between clinical and genetic diagnosis is acceptable, a genetic test like this could aid in the methodology of diagnosing dogs, as well as being a useful tool in finding out more about the prevalence of the disease. Knowledge of the genetic prevalence of CRD and information about the compliance between clinical and genetic diagnosis would be useful for veterinarians, dog breeders and dog owners.

Aim

CEA is a widespread genetic eye disorder affecting multiple breeds, but especially the Collie. In Denmark, CEA is found in about 70% of the Collies examined (information obtained from the Danish Collie Club). This thesis is partly based on the previous work of Parker *et al.* (2007), who found a mutation in a possible candidate gene for CRD. By investigating DNA samples from clinically affected and unaffected Rough Collies, we hope to increase the knowledge of the genetic prevalence of CRD in the Danish Rough Collie. We will investigate the following questions:

1) Is there compliance between the clinical and genetic diagnosis of CRD?

2) Can genetically unaffected Rough Collies be identified in the Danish population?

Delimitation

A literature study has been performed to account for the clinical and genetic background of the disease. Literature written from 1965-2014 has been used. In addition to this, a genetic case-control study was performed on Danish Rough Collies.

The project was carried out as a 30 ECTS master's thesis. This short amount of time has obviously limited the magnitude of the project. We have collected DNA samples from 45 Rough Collie dogs, but it would have been useful and interesting to collect DNA samples from more dogs, as well as including the other affected breeds in the study.

Method

In this study, two different methods are used. A literature review was conducted in order to highlight previous research and its results. Scientific articles, books and websites have been used to collect information. Secondly, a genetic case-control study was carried out with the ambition to find clinical and genetic compliance. Furthermore, the aim was to evaluate whether there are any genetically unaffected dogs in the Danish population of Rough Collies. The 45 Collies enrolled in the study were appointed by the Danish Collie Club. All these dogs were unrelated at a parental level, representing ophthalmoscopically examined dogs, with and without a CEA diagnosis. Buccal swabs were collected from the dogs by the owners and sent to the laboratory; the DNA was isolated and PCR was performed with specific primers for the mutation in the *NHEJ1* gene.

Relevant Anatomy of the Eye

The eyeball consists of three layers:

- **Fibrous layer** – sclera and cornea
- **Vascular layer** – choroid, ciliary body and iris
- **Inner layer** – nonvisual retina and optic part of the retina

The eyeball encloses the interior chambers of the eye, which are:

- Anterior chamber
- Posterior chamber
- Vitreous chamber (vitreous body)

Sclera

The major part of the sclera is made up of a network of collagen fibres, contributing to the shape of the eye. Elastic fibres are interspersed among the collagen fibres, helping to resist the pressure from the internal parts of the eye and the forces by the extraocular muscles. The sclera also plays an important role in controlling the ocular pressure by draining the aqueous humour (the fluid in the anterior and posterior chamber). A defect in the draining of the aqueous humour can lead to an increased ocular pressure, which might cause glaucoma.

Choroid

The choroid is a pigmented and highly vascularized layer that is responsible for supply of nutrition to the retina. The tapetum lucidum is a half-moon-shaped area in the choroid. Over the area of the tapetum lucidum the retina is normally pigment-free. This enables incoming visible light to be reflected back to the retina, improving night vision. In the dog the tapetum lucidum has a distinctive green colour.

Retina

The retina is the innermost layer of the eyeball, and consists of a nonvisual and an optic part. The nonvisual part is composed of an outer and an inner layer. The outer layer is pigmented but the inner layer is unpigmented. The nonvisual part of the retina lines the anterior of the eye, forming the posterior border of the iris.

The optic part of the retina is considerably thicker than the nonvisual part, and lines the posterior part of the eye. The optic part is also composed of two layers, the pigmented layer and the neural layer. The pigmented layer is the outermost layer of the retina, neighbouring the choroid. As mentioned above, the choroid is the main supply of nutrition to the retina, but retinal arteries supply some parts of the retina. The pigmented layer of the retina, together with the pigmented parts of the choroid, aids vision by absorbing light as well as reducing scatter and enhancing contrast. The neural layer is the innermost layer, and this layer holds the photoreceptors cells, namely the rods and cones. This optic part of the retina is the part of the eye where photic energy is transduced into chemical energy and finally into electrical impulses. These electrical impulses are then transmitted to the visual centres of the brain by the optic nerve. The optic disc is the point in the eye where the axons of retinal ganglion cells come together to form the optic nerve. In this area there are no rods or cones, and the area is therefore commonly referred to as the blind spot.

Retinal detachment, which can lead to serious consequences, most often happens along the space between the pigmented epithelium and the neural layers of the retina.

Optic nerve

The optic nerve, also called the second cranial nerve, is about 2 mm in diameter in the dog. The nerve is made up of axons of the multipolar cells from the ganglionic layer of the retina. Up until they collect at the optic disc, the axons are unmyelinated, but become myelinated hereafter when passing through the cribrosa of the sclera, forming the optic nerve.

At the optic chiasm, the majority of the nerve fibres cross over to the contralateral side, and then continue on to the visual area of the cerebral cortex as well as to the supraoptic and paraventricular nucleus of the hypothalamus (König *et al.* 2009).

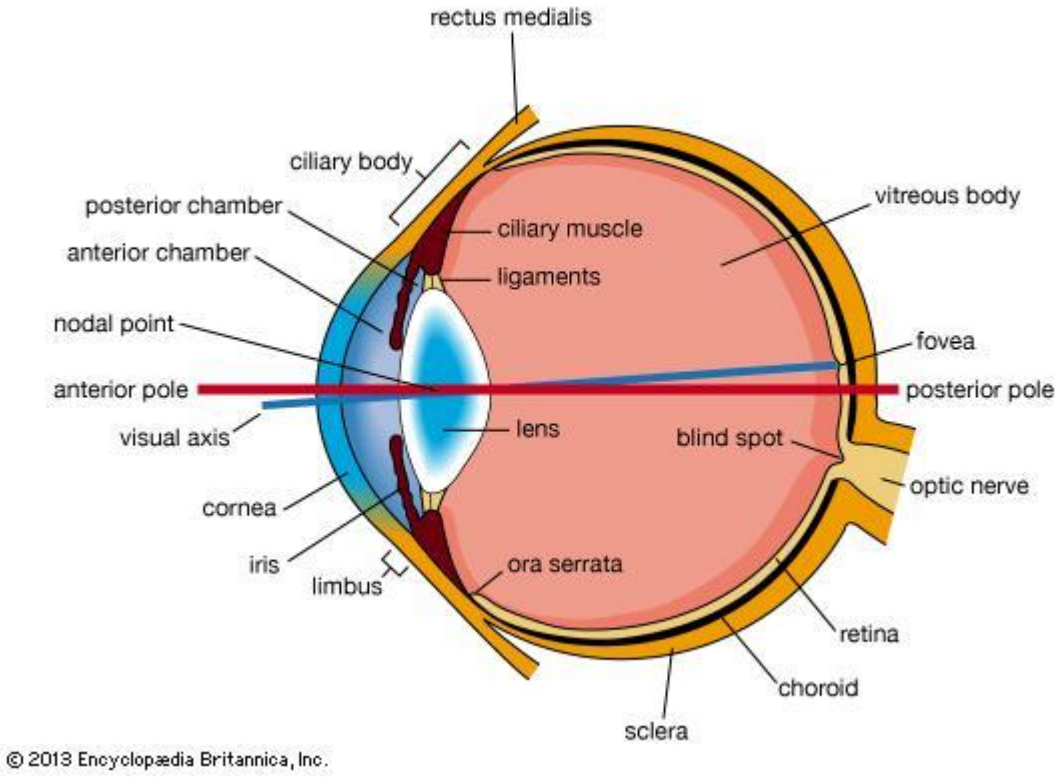


Figure 1. A schematic picture of the eyeball in section (http://global.britannica.com/EBchecked/topic/1688997/human-eye).

Clinical Pathology of CEA

CEA is a congenital eye abnormality affecting the posterior part of the eye, and involves the sclera, choroid, retina, tapetum, optic nerve head and ocular blood vessels. CEA is a non-progressive, bilateral disease. However, the degree of defect in the two eyes of an affected individual is often dissimilar. The disease manifests itself in various ways between individuals; it ranges from mild, with no effect on vision, to severe, with total blindness. CEA includes two basic abnormalities – chorioretinal dysplasia and coloboma. Secondary changes, such as retinal detachment and intraocular haemorrhage, may also be present (Barnett 1979; Bedford 1982b).

Clinical Features

Chorioretinal Dysplasia

Chorioretinal dysplasia (CRD), sometimes described as chorioretinal hypoplasia (Bedford 1982b), is the primary abnormality in CEA, but has the least effect on vision. It has been referred to as the ”pale area”, which well describes that CRD is an area of poor

pigmentation, i.e. an area in the retina and/or choroid which is poorly developed or absent of pigment. In its mild form a small area is affected, whereas in the more severe form a larger area is affected, sometimes incorporating the optic disc (Barnett 1979).

Ophthalmoscopically, findings of CRD vary to a great extent. The findings can range from an absence of retinal and choroidal pigment and tapetal hypoplasia to malformations and absence of choroidal blood vessels (Bedford 1998). The malformations of the choroidal blood vessels are described by Barnett (1979) as a widening of the vessels.

The abnormality is congenital and can be diagnosed with certainty in young puppies, 5-10 weeks old, using ophthalmoscopy. This defect is non-progressive, and therefore, does not change in appearance or severity throughout an individual's life (Barnett 1979). However, the reason that CRD must be diagnosed before the age of 10 weeks is that the retina changes colour from blue to yellow/green shortly thereafter (Bjerkås 1991). This change in retinal pigmentation can mask minor chorioretinal changes, and the phenomenon, when affected dogs appear normal, is called "go normal" (Bedford 1982b). CRD is more difficult to diagnose in dogs with the coat colour blue merle, when located in the subalbinotic fundus. However, the typical position of CRD in the temporal part of the optic disc, as well as the presence of abnormal choroid blood vessels, aids diagnosis.

On a histopathological level, tapetal cells are missing, pigment in the pigment epithelium and choroid is lacking, and the choroid is not as thick as in a healthy eye (Barnett 1979).

Coloboma

Some dogs with CRD are affected by another abnormality as well, namely coloboma. Colobomas can sometimes be present without CRD, but this seems to be very rare (Walser-Reinhardt *et al.* 2009). Although less common than CRD, a coloboma is a much more severe abnormality. This defect causes pits in the optic disc or its surrounding region, which in turn affects vision. Depending on the depth and size of a coloboma, vision is affected to different degrees.

In dogs with CEA, colobomas vary from being very small and shallow, affecting only a part of the optic disc, to being much bigger and causing the area of the optic disc to become enlarged. In some cases colobomas can cover a large area at the posterior pole of the eye, which causes the optic nerve to be pushed to one side (Barnett 1979).

Secondary Changes

A series of secondary changes may be present in dogs affected by CEA. The most important of these is retinal detachment, as it has a big impact on vision (Barnett 1979). Retinal detachment most often occurs in combination with colobomatous defects in the area of the optic nerve (Roberts *et al.* 1966). The retina most often detaches during the first twelve months of a dog's life, but it can occur later in life as well. Detachment is more easily observed using indirect opthalmoscopy, compared to direct, due to its stereoscopic field of view. When first diagnosed, a detachment may be partial or total, but even a partial detachment can later in life develop into a total detachment. Haemorrhage in the vitreous can occur together with retinal detachment, but it is not seen in all cases. Another cause of intraocular haemorrhage may be arteriolar loops. Intraocular haemorrhage often occurs unilaterally and can cause defective vision and, sometimes, total blindness.

Tortuous vessels, especially tortuosity of the primary retinal veins, are a finding that sometimes is described as a sign of CEA. However, the Collie has a higher degree of tortuosity of retinal vessels in the normal fundus compared to other breeds, especially in the arterioles. This can make it difficult to diagnostically differentiate between normal tortuosity and excessive tortuosity (Barnett 1979). Some authors do not consider tortuous vessels to be a relevant sign of CEA (Bedford 1982a).

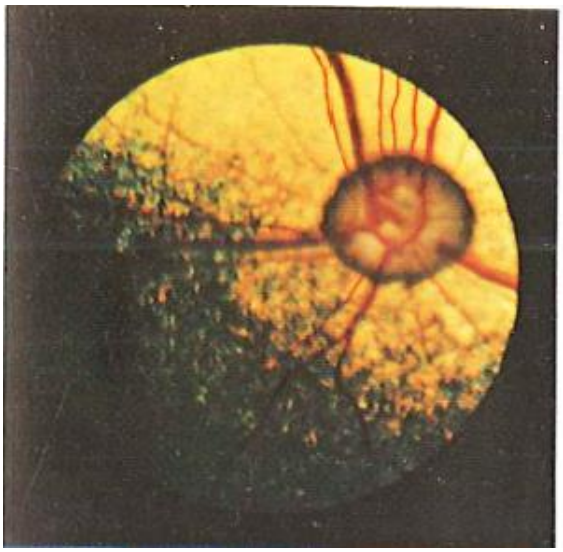


Figure 2.
Normal Collie fundus (Donovan and Wyman 1965).

Figure 3.
CRD Rough Collie (Bedford 1982b).

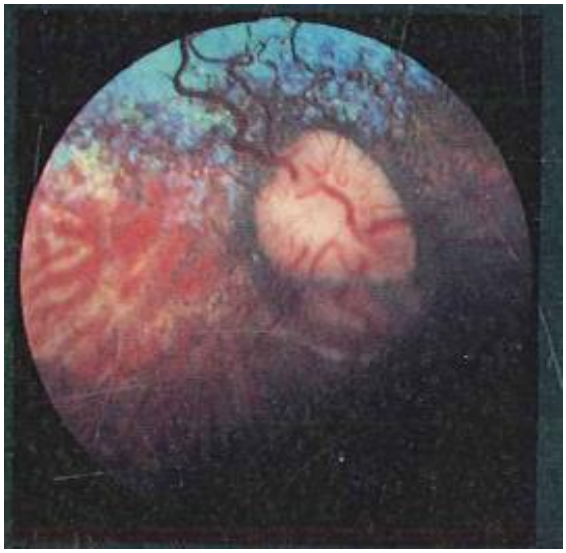


Figure 4. Coloboma Rough Collie (Bedford 1982b).

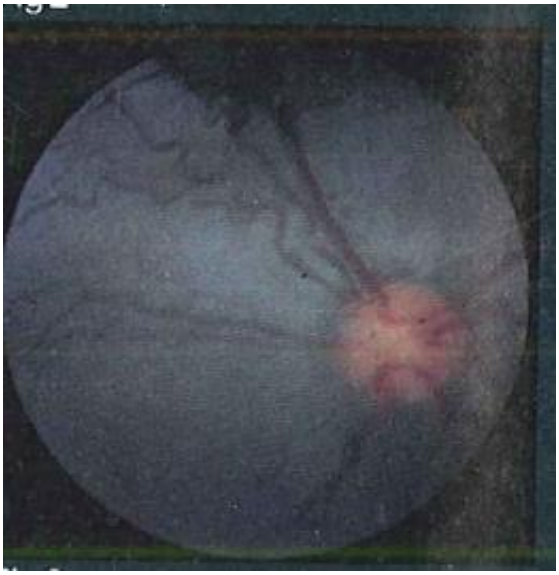


Figure 5. Normal fundus in a Rough Collie puppy, six weeks old (Bedford 1982b).

Embryology and Pathology

The pathogenesis of CEA seems to be a defect in the inductive chemical signaling from the developing retinal pigment epithelium (RPE). The inductive failures cause insufficient stimulation of the periocular mesenchyme (later forming the choroid and the sclera), which in turn causes hypoplasia and hypopigmentation of the choroid. Another manifestation of the disease is delayed closure of the optic fissure, which is caused by arrested growth of the fibrous tunic of the sclera. This makes it possible for the retina to bulge out of the optic fissure, which, in turn, prevents the complete closure of the embryonic fissure and sclera. This defect produces a scleral ectasia, and is called posterior polar coloboma, or optic disc coloboma.

In addition to what is described above, CEA can also include mild micropthalmia, congenital retinal folding, tapetal hypoplasia, and retinal detachment. As opposed to most of these defects, retinal detachment is not present at birth, but is most commonly delayed until the puppy is a few months old. Just like choroid hypoplasia, the micropthalmia and retinal folding are believed to be caused by improper signaling from the RPE. If the growth rate of the retina exceeds that of the scleral shell, this will cause the excess retina to fold. However, these folds can eventually disappear, as the scleral and retinal growth normalizes. As opposed to this scenario, if the growth of the retina is deficient while the growth of the sclera continues normally, a stretch of the retina will occur, and this can eventually cause complete retinal detachment (McGavin *et al.* 2007).

Clinical Diagnosis

As mentioned above, the age of diagnosis is of great importance due to the fact that the retina becomes pigmented, and there is a risk of missing the window of opportunity to diagnose individuals that later will “go normal” (Bedford 1982b). The recommended age of diagnosis vary slightly between authors, but generally it is agreed that diagnosis should be done before the age of 10 weeks. According to Donovan (1969), the optimal age for diagnosing CEA/CRD is between 5 and 6 weeks, but it can be done as early as 3 weeks of age.

It should be mentioned that even when examining a puppy before 10 weeks of age, it can sometimes be challenging to diagnose CEA as the normal Collie fundus can vary greatly in appearance (Barnett 1979). Other factors that can make it difficult to diagnose young puppies is that they have small eyes, and their playful temper can make it difficult to hold them still for a sufficiently long time (Bedford 1980). Coat colour can also complicate diagnosis. Typically the blue merle with albinotic fundi has been described as more difficult to diagnose (Yakely 1972).

Prior to examination the pupils need to be dilated, which is done using a short acting mydriatic. The ophthalmoscopic examination then takes place in a dark room. To diagnose CEA, binocular indirect ophthalmoscope is the most suitable equipment. Compared to direct ophthalmoscopy, indirect ophthalmoscopy allows larger area of the fundus to be examined (Donovan 1969). In order to make the examination as thorough as possible, it is advisable to use direct and indirect ophthalmoscope (Möller 2014, personal communication).

Figure 6. Ida Möller examining a Shetland Sheepdog puppy using direct ophthalmoscope.

Figure 7. Ida Möller examining a Shetland Sheepdog puppy using indirect ophthalmoscope.

Genetics

Since the disease was first described by Magrane in 1953, a lot of speculation concerning the mode of inheritance has taken place. The fact that CEA is a genetic disease was suspected early on, based on the fact that inbreeding increased the prevalence and outbreeding diluted it (Donovan & Wyman 1965). To this day, it seems that there is no total agreement between experts regarding the exact mode of inheritance of CEA. One of the leading groups of researchers on the subject, believe that CEA is inherited as a polygenic complex syndrome (Wallin-Håkanson *et al.* 2000a and b), and that CRD and colobomas are inherited as separate traits. Wallin-Håkanson *et al*. (2000a and b) received interesting comments on their work by the lecturer David R. Saragan in 2001. Dr Saragan described how their findings could be explained by CEA having modifier gene loci and incomplete penetrance. By discussing this, Dr Saragan was trying to argue that their results could be interpreted as CEA having a simple autosomal recessive mode of inheritance and that there is hope for the development of a genetic test for the disorder by finding a gene of "major effect". However, in their response, B. and N. Wallin-Håkanson politely argue that Dr Saragan confirms that CEA seems to be polygenic, by bringing up the concepts of modifier gene loci and incomplete penetrance. They go on to discuss that their results strongly suggest that CRD and colobomas are inherited separately, and that they believe CEA to be polygenic. They believe that several additive or otherwise interacting gene loci are involved in the genetics of CEA (Sargan & Wallin-Håkanson B. and N. 2001).

Non-genetic factors, such as environmental factors, have been discussed to influence the various ways in which the anomaly manifests itself (Yakely 1972). Data has shown that there is no significant difference between the occurrence of the anomaly in male and female dogs, suggesting that it has an autosomal inheritance (Yakely *et al.* 1968). Most of the genetic studies involve CRD and coloboma, while the secondary changes are rarely mentioned.

The mode of inheritance of CRD, the primary phenotype of CEA, has also been under discussion. Some authors believe CRD to be inherited as a simple autosomal recessive trait (Donovan *et al*. 1969). Others have conducted studies with results suggesting that CRD is

inherited as a polygenic trait (Wallin-Håkanson *et al.* 2000b). Attempts have been made to genetically map CRD. In 2003, the locus of CRD was mapped, using segregation studies, to a 3.9-cM region on canine chromosome 37 (CFA37) by Lowe *et al.* (2003). Although they concluded that the phenotype is caused by a single allelic mutation, they discuss the possibility that their mapping might have been obscured by independently segregating modifier loci, as they believe that CRD might be a complex trait with more than one genetic contributor. Their studies suggest that the penetrance of CRD is less than 100%, with an incomplete penetrance in homozygous affected dogs and, noteworthy, partial penetrance in heterozygous dogs (carriers) (Lowe *et al.* 2003). In recent years it has been found that all dogs affected by CRD share a homozygous intronic deletion of 7.8 kbp in the *NHEJ1* gene (Parker *et al.* 2007), which we will describe more in depth under genetic mapping of CRD.

Studies indicate that colobomas have a different mode of inheritance than CRD, and are suggestive of a dominant autosomal mode of inheritance. It has been reported that colobomas only penetrate in dogs homozygous for CRD (Donovan *et al.* 1969), however, even if it is rare it seems that colobomas can be present without CRD (Walser-Reinhardt *et al.* 2009). In accordance with Donovan´s (1969) findings, Wallin-Håkanson *et al.* (2000a) have found that the prevalence of colobomas did not increase as the prevalence of CRD increased over time in a Collie population where more and more CRD-affected dogs were used in breeding. Again, this suggests that colobomas are inherited separately from CRD (Wallin-Håkanson *et al.* 2000a).

Interestingly, it has been found that vitality is affected in dogs with CEA when colobomas are involved. Studies have shown that matings between dogs with colobomas and dogs affected with CRD and/or colobomas lead to smaller litter sizes, compared to matings between healthy individuals and between two CRD affected individuals (Wallin-Håkanson *et al.* 2000b). Since diagnosis of CEA is possible as early as at 3 weeks of age (Donovan *et al.* 1969), it can be speculated that breeders who discover that they have CEA affected puppies early on choose to euthanize those individuals before the “official” examination takes place. If this occurs, the numbers used in the study by Wallin-Håkanson *et al.* (2000b) would be unreliable.

Genetic Mapping of CRD

After Lowe *et al.* (2003) had localized the CRD phenotype to a region on CFA37; new studies were set up in order to find the genetic mutation causing CRD. In the beginning of their article, Parker *et al*. (2007) declare that it can be difficult to specify which exact genetic sequence that is responsible for a certain phenotype. In their attempt to find the primary genetic mutation causing CRD, cluster analysis was being used. They assessed population structure across breeds, using 137 dog breeds. The breeds were divided into five primary breed groups, where the breeds in every group probably have common ancestors and, therefore, share certain traits. One of the clusters recognized was the herding/sighthound group, containing 24 breeds. The Collie, Shetland Sheepdog, Border Collie and Australian Shepherd were included in this group, all of which are known to be affected by CEA, which led to the suspicion that they might share a causative mutation identical by descent.

Extensive work was done, utilizing the human homologous region to CFA37, the 7.5x Boxer sequence assembly (CanFam1), and a SNP database, in order to design new markers for the fine mapping of CRD. By testing several SNPs, the shared haplotype area was reduced to 102,495 bp. A canine BAC contig map was constructed across the candidate interval. This map enabled them to find that the entire linkage disequilibrium (LD) interval was included in a single BAC clone, by using PCR analysis with SNPs and other markers. The LD interval was defined by a common SNP haplotype among all tested CRD affected chromosomes. Four genes and a non-coding region were identified within this LD interval. Sequence analysis was performed, and no candidate for the causative mutation was found among the exons and flanking regions of the shared haplotype. An intronic mutation was detected in all affected chromosomes tested, that potentially could be significant. The mutation was a 7799-bp deletion within the gene *NHEJ1*.

The deletion needed to be investigated further, so a two-step PCR test was set up to estimate its occurrence, using samples from multiple breeds. The PCR test was based on two sets of primers, placed inside and outside the deletion, allowing quick identification of chromosomes with and without the deletion.

90 dogs from four breeds were tested; most of which were known to be affected by CRD, and some obligate carriers. Another 93 dogs were tested, but these dogs were from 45 breeds not known to segregate CRD. In all CRD affected dogs the deletion was present in both chromosomes, while it was present only in one chromosome in the obligate carriers. The deletion was found in only one chromosome of the 93 dogs from unaffected breeds, a Boykin Spaniel. This heterozygous Boykin Spaniel was presumed a carrier, and 16 other Boykin Spaniels related to that individual were tested. Another five individuals, heterozygous for the mutation, were found and later a few other Boykin Spaniels, segregating the CRD phenotype, were confirmed to also be homozygous for the deletion.

Several other breeds known to be affected by CRD, or CRD-like phenotypes, which had not been included in the study so far, were tested for the 7799-bp deletion. The deletion was found in the Lancashire Heeler, Nova Scotia Duck Tolling Retriever and Longhaired Whippet breeds, and all CRD phenotypically affected dogs were homozygous for the deletion while obligate carriers were found to be heterozygous. Two dogs clinically diagnosed with colobomas, from the breed Berger des Pyrenees, were tested for the deletion, but it was not present. Finally, a few Soft Coated Terriers, known to be phenotypically affected by lesions similar to CRD, were tested, but the deletion was not present in any of these terriers.

The 7799-bp deletion is located within intron 4 on the *NHEJ1* gene, and comparative genome analysis has shown that the sequence partly comprises highly conserved elements. Furthermore, this genetic sequence contains binding sites for several regulatory proteins, and it is believed that the deletion affects ocular development of the mammalian eye. Parker *et al*. (2007) go on to discuss that their work indicates that the deletion arose as a single disease allele, and that it was spread to a wide range of herding dogs a very long time ago when dog breeds were developing.

Some of the breeds in which the mutation in the *NHEJ1* gene was found, do not seem to have a clear connection to the Collie or other herding breeds. Both the Nova Scotia Duck Tolling Retriever and the Boykin Spaniel cluster with the hunting breeds. The fact that they share the *NHEJ1* mutation with many of the herding dogs, indicate that they do have common ancestors. It is difficult to know exactly how dog breeds were established, but it has

been reported that the Nova Scotia Duck Tolling Retriever has ”farm Collies” among its ancestors (Parker *et al*. 2007).

Genotyping using conventional PCR

The DNA test for CRD is based on DNA amplification using a technique called polymerase chain reaction (PCR). In 1985, this method of isolating and amplifying large amounts of specific DNA fragments was developed. PCR takes place in a thermal cycler, in which the temperature of incubation changes in a programmed manner in three steps.

In order to amplify the wanted region of DNA, specific oligonucleotides of 16-26 nucleotides, whose sequence match the sequence of a flanking region to the target DNA region. These specific oligonucleotides are called primers, and their design is of great importance for the reaction to amplify the correct region of the DNA sample. Each PCR acquires two primers; one complementary to one of the DNA strands at one end of the target region, and the other primer is complementary to the other DNA strand at the other end of the target region. In addition to the DNA sample and the two primers, a solution of the four deoxynucleotides and a specialized polymerase, called *Taq* DNA polymerase, are needed. The test tubes containing all these components are then placed in the thermal cycler.
During the first step the temperature raises to around 94°C for 5-15 minutes, and the DNA strands are separated into single strands (denaturation). During the second step the temperature is lowered to 50-60°C for 30 seconds, and at this temperature the primers pair up with the complementary sequences in the single stranded DNA (annealing). The temperature, and sometimes the time, of this step vary, depending on the GC: AT ratio and the length of the primers. During the third step the temperature raises to around 72°C, which allows the *Taq* polymerase to function, leading to polymerization of DNA to proceed (extension). The time of this step varies between 1-5 minutes, and depends on the length of the target sequence.

When the third step is over, the amount of target DNA is doubled, and from here the thermal cycler starts over, and continues for as many cycles as programmed to (20-40).

During these subsequent cycles the first step only lasts for around 30 seconds. The reason the time of the first step can be reduced is that the newly formed DNA stretches, containing one of the original strands and one newly synthesized complementary strand, are rather short. As the cycling continues, DNA strands of the target region will be generated exponentially (Hartwell *et al.* 2011). Figure 8 shows the three steps of the PCR.

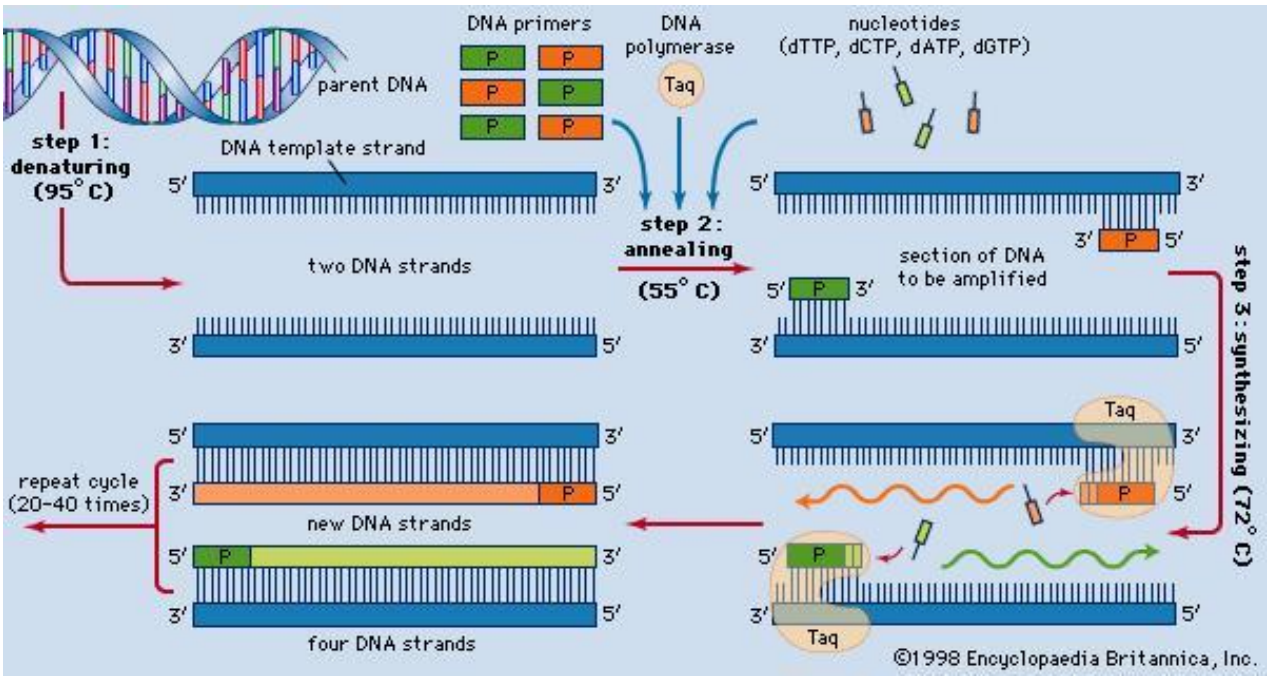


Figure 8. Illustration of the three-steps in the PCR-reaction (http://global.britannica.com/EBchecked/topic/468736/polymerase-chain-reaction).

Breeding Recommendations

Despite the fact that CEA is a congenital hereditary disorder, there are no breeding restrictions for the Collie breed in Denmark, stating which individuals are allowed to be used in breeding. Nonetheless, since 2006 it has been a requirement to ophthalmologically examine the parent dogs (nothing is written about when this examination should take place) in order to get your puppies pedigreed (collie.dk). This means that the clinical status of the parent dogs must be registered, but even if a dog is found to have a severe form of CEA, there is nothing that prevents the owners from using that dog in breeding. Hence, it is of great importance that veterinarians performing the ophthalmic examinations enlighten the owners of up to date breeding recommendations. Hereafter, one can only hope that the breeders have enough concern for the health of the breed to take the recommendations sternly.

The Danish Kennel Club refers to the breeding recommendations for CEA that the Danish Society for Veterinary Ophthalmology have published on their webpage (Dansk Kennel Klub). The recommendations are that dogs with CRD should only be mated with dogs that are unaffected by CEA, and dogs with colobomas or secondary changes (retinal detachment etc.) should be excluded from breeding (Dansk Selskab for Veterinær Ophthalmologi 2013).

Maintaining a relatively large gene pool in a population is of great importance. Inbreeding decreases the gene pool, and this elevates the risk for recessive conditions to increase in number (Strachan & Read 2010). As mentioned by Wallin-Håkanson *et al.* (2000a), Collie breeders are known to have used CEA affected individuals for breeding in order to maintain a sufficiently large breeding population. Since the number of affected Collies is so large, it is impossible to completely exclude all CEA affected Collies from breeding. Consequently, breeder awareness, breeding programs based on breeding recommendations, and central registration of CEA status are crucial factors in trying to reduce the breed's problems with the anomaly. Already in 1972 it was acknowledged how important it is to include phenotypically normal dogs in breed-improvement programs, as well as recording the CEA status of all individuals (Yakely 1972).

In the Czech Republic they have conducted a study where the DNA of 379 dogs of different breeds was analysed for the mutation in the *NHEJ1* gene. It was found that the allele frequency of the mutation was by far the highest in the Rough Collie (0.797). The authors discuss ways of lowering this high allele frequency without losing other valuable traits in the Collie. Since the number of homozygous healthy Collies is so low, their suggestion is to start by mating carriers with carriers, producing about 25% healthy, 50% carriers and 25% affected offspring. If then continuing to genetically test all individuals, more and more homozygous healthy dogs can be used in breeding, and eventually there will be more genetically healthy dogs in the Collie population (Dostál *et al.* 2010).

Materials and Method

Materials

For this study, dogs of the breed Rough Collie who met two criteria were included:

1) Diagnosed before the age of 10 weeks
2) Unrelated on a parental level (i.e. no siblings)

The Danish Collie Club selected approximately 100 Rough Collies that fulfilled the criteria, and the owners of these dogs were asked to be part of this study. A description of the study was sent to them by mail (see Appendix 1) together with a guideline of how to collect DNA samples from the buccal mucous membrane on FTA cards (see Appendix 2). Unfortunately only 45 owners chose to participate in the project, and the FTA cards from their dogs were sent to the Section for Animal Genetics, Bioinformatics and Breeding, at the Institute for Clinical and Veterinary Sciences. Out of the samples received, 11 were taken from clinically healthy dogs, 31 diagnosed with CRD and 3 diagnosed with CRD and coloboma (see Appendix 3).

Method

Isolation of total DNA

We isolated total DNA from our sampled buccal cells on the FTA cards, using the protocol from QIAamp® DNA Investigator Handbook. For a detailed description see Appendix 4.

Polymerase Chain Reaction

Primer Design

In order to find the deletion in gene *NHEJ1*, two primer sets were needed. The primer sets we needed were designed by Parker *et al.* (2007):

Within deletion:

NHEJ1-F17, 5´- TCTCACAGGCAGAAAGCTCA-3´

NHEJ1-F17, 5´- CCATTCATTCCTTTGCCAGT-3´

Across deletion:

NHEJ1-F20, 5´- TGGGCTGGTGAACATTTGTA-3´

NHEJ1-R23, 5´- CCTTTTTGTTTGCCCTCAGA-3´

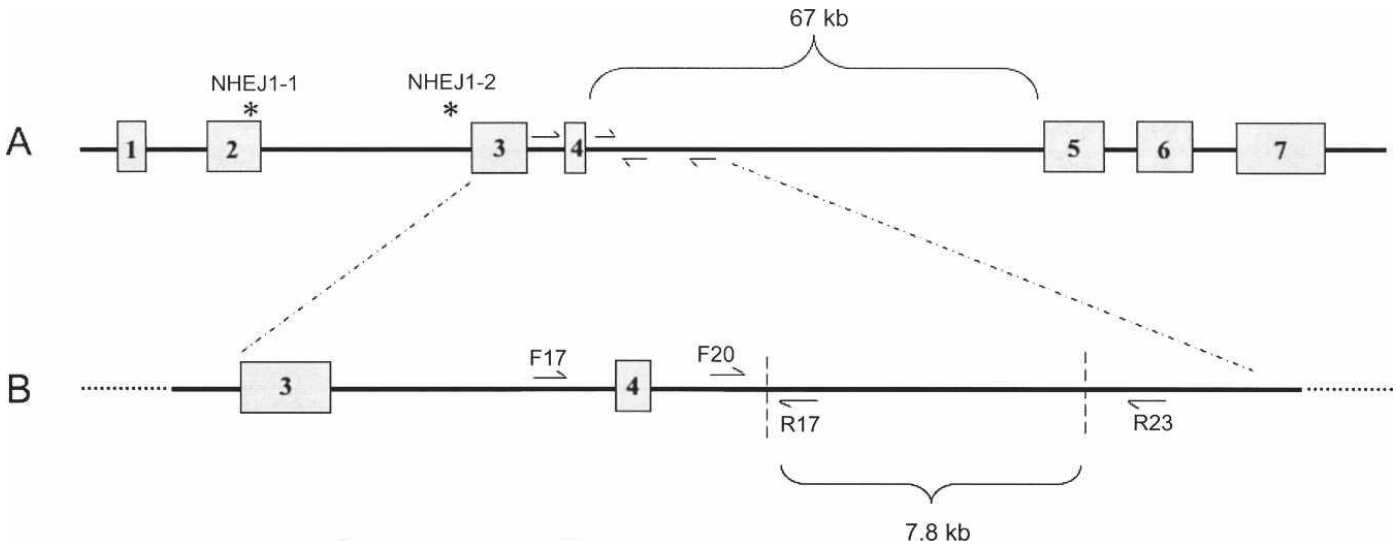


Figure 9. This figure demonstrates how a two-step PCR protocol is used for genotyping the CRD-associated deletion. A) A schematic image of the *NHEJ1* gene with two linked SNPs (designated by *), and primer locations within intron 4. B) An expanded representation of the CRD-associated region, with flanking and internal primers.

PCR Reaction

In our PCR reaction the mastermix contained the following:

1,0 µl 10xbuffer (1,5 mM $MgCl_2$)
0,5 µl dNTP
0,35 µl primer F
0,35 µl primer R
0,05 µl *Taq* polymerase
6,75 µl H_2O

9,0 µl mastermix together with 1,0 µl DNA were put in the PCR test tubes, i.e. 10 µl in total.

The following PCR program was used:

1: 94°C i 15 min (activates *Taq* polymerase)
2: 94°C i 30 sek (denaturation)
3: 60°C i 30 sek (annealing) } x 35
4: 72°C i 45 sek (extension)
5: 72°C i 10 min (extension)
6: 4-12°C (storage temperature)

Gel Electrophoresis

Gel electrophoresis was the method used to separate our DNA fragments. Electrophoresis works by passing an electrical current through agarose gel, and the different fragments separate dependent on size (Hartwell *et al.* 2011). A 2% agarose gel was used. We compared size with the markers 100 base pairs (bp) and 1000 base pairs (1 kbp). In one of the wells we used water as a blank.

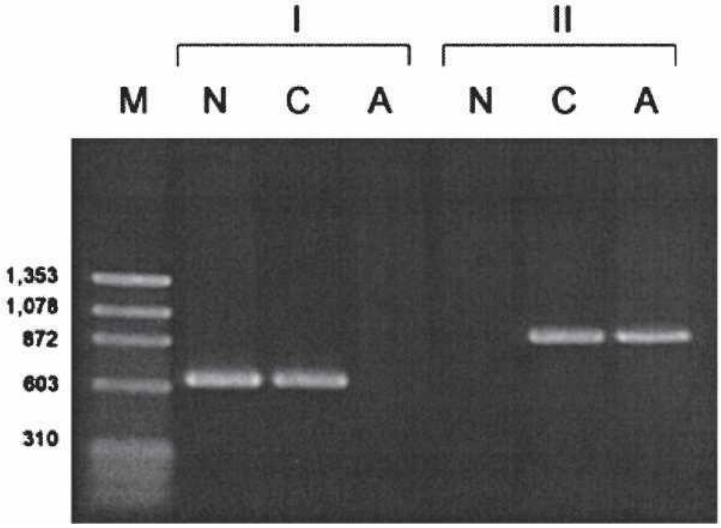


Figure 10. Electrophoretogram demonstrating amplification results using primers *NHEJ1*-F17-*NHEJ1*-R17 (I) and primers *NHEJ1*-F20-*NHEJ1*-R23 (II). (M) marker, (N) normal, (C) carrier and (A) affected.

Throughout the entire procedure of genotyping the DNA samples from the 45 Collies, we were unaware of which individuals were phenotypically affected by CRD and which were phenotypically healthy, i.e. the study was blinded.

Results

Out of the 45 dogs that were genotyped, only one dog, CEA021, which clinically had been diagnosed as non-affected, appeared to be a genetic carrier of CRD, i.e. heterozygous (*CRD/crd*). All the other 44 dogs appeared to be genetically affected, meaning they were homozygous for the mutation in the *NHEJ1* gene (*crd/crd*). None of the dogs were genetically healthy, i.e. homozygous for the wildtype (*CRD/CRD*).

		Genotype frequencies			Allele frequencies	
Breed	n	Homozygous for wildtype *CRD/CRD*	Heterozygous *CRD/crd*	Homozygous for mutation *crd/crd*	Wildtype allele *CRD*	Mutation allele *crd*
Rough Collie	45	0	1	44	0,01	0,99

Table 1. Genotype and allele frequencies of the gene *NHEJ1* in Rough Collies. The wildtype ("healthy") allele is labelled *CRD*, and the mutant ("affected") allele is labelled *crd.* Out of the 45 dogs, 11 were diagnosed as clinically healthy, 31 were clinically diagnosed as affected by CRD and 3 by CRD + coloboma, see Appendix 3.

100 bp	1 kbp	CEA 002	CEA 003	CEA 004	CEA 005	CEA 006	CEA 007	CEA 008	CEA 009	CEA 010	CEA 011	DDG 118	H_2O

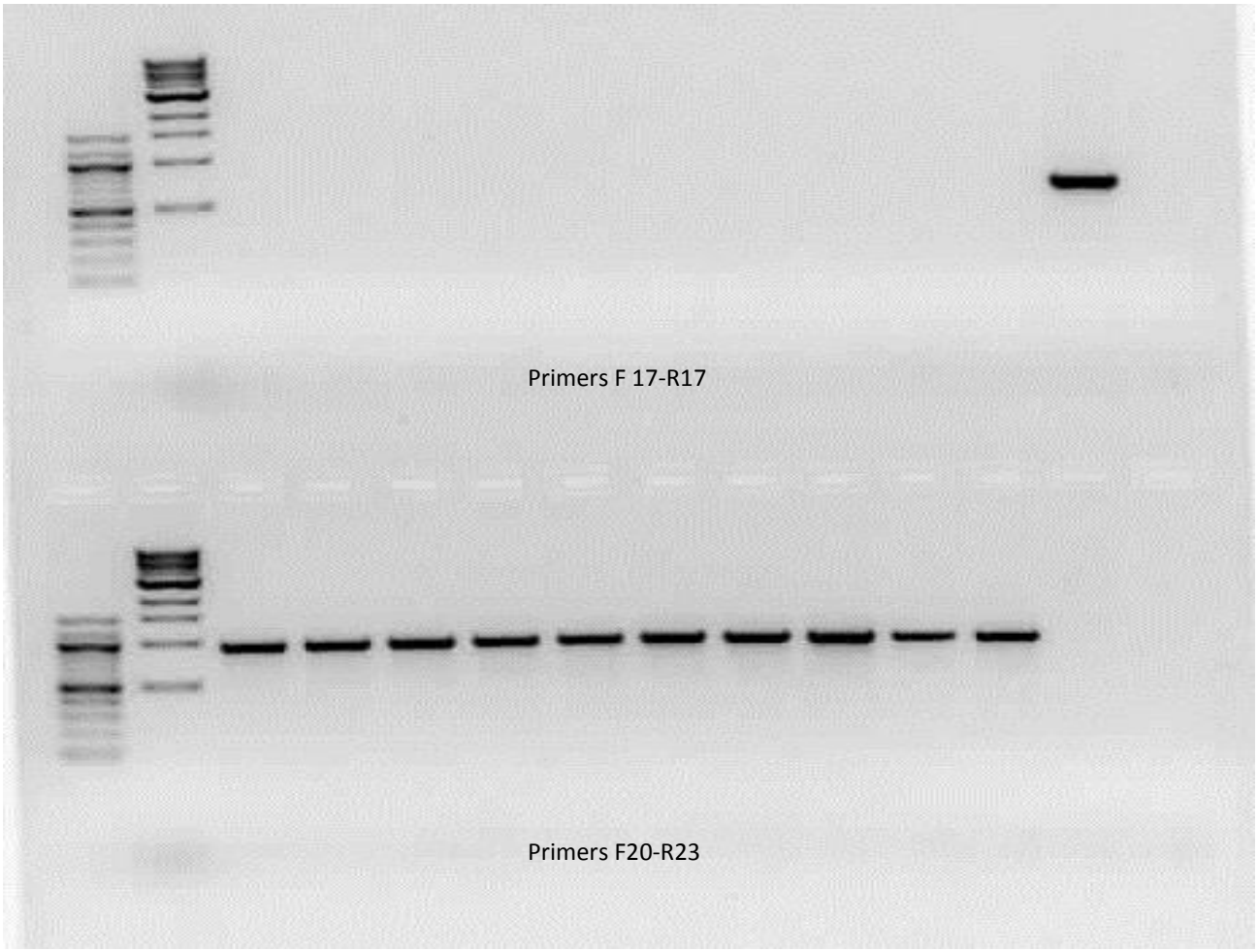


Figure 11. Electrophoretogram demonstrating PCR results from dogs CEA002-011, a control dog DDG118 and a blank H_2O, using the two primer sets F17-R17 and F20-R23. This should be interpreted as the control dog being homozygous for the wildtype allele (*CRD/CRD*), and all the other dogs, CEA002-011, being homozygous for the mutant allele (*crd/crd*), i.e. genetically affected by CRD. Electrophoretogram of the remaining dogs can be found in Appendix 5. The clinical status for all dogs can be found in Appendix 3.

Discussion

The results obtained in our study show that 10 out of 11 clinically healthy dogs appeared to be homozygous for the deletion in the *NHEJ1* gene, i.e. genetically affected. Only one of the clinically healthy dogs turned out to be heterozygous, in other words a carrier of the deletion in the *NHEJ1* gene. All the dogs diagnosed as clinically affected turned out to be genetically affected as well, meaning they were homozygous for the deletion in the *NHEJ1* gene. This means that the compliance in our study between genetic and clinical diagnosis in the clinically affected dogs is total, while the compliance in the clinically healthy dogs is very poor. We might not have expected the compliance between the clinical and genetic diagnosis to be perfect, but we did expect it to be better than it turned out to be.

The method used in this study was straightforward and effective. It could be argued that the owners should not have sent in the buccal swabs themselves, but rather come in to the clinic for us to take the swabs. That way, there would have been no doubt that each DNA sample came from the correct dog. Even though we bring up this as one part of the method that could be improved, we do not consider it to be a big problem in this study, as we do not think the owners had much reason to take the swabs from incorrect dogs.

One obvious limitation of our study is the number of dogs included. It would have been profitable to include a much higher number of dogs, and dogs of all breeds known to be affected by CEA could have been included. The clinical examinations should take place one time before the age of 10 weeks. If carrying out a more extensive study, it would also be interesting to have complete pedigrees of all dogs as far back as possible, informative of the CEA status of as many individuals as possible. Furthermore, it would be interesting to genetically test individuals from the same litter as well as all their living forefathers and foremothers, even if a clinical diagnosis was not done before the age of 10 weeks in all individuals. This would give a closer view of how the genetic mutation segregates, alongside with how the phenotype segregates.

When it comes to diagnosing CEA, there are factors, as described above, which can make a clinical diagnosis uncertain. The fact that CRD in some cases can be difficult to diagnose, forces us to take into consideration that some of our clinically healthy dogs could be misdiagnosed, i.e. false negative. It is, however, unlikely that 10 out of 11 dogs would be misdiagnosed. In addition, it is very unlikely that any of the dogs in this study would be "go normals", as all the dogs were diagnosed before the age of 10 weeks. The "go normal" phenomenon could otherwise have been a reasonable explanation for the poor compliance obtained between the clinically healthy individuals and the genetic diagnosis.

Since Parker *et al*. (2007) found the mutation in the *NHEJ1* gene, it seems that no one has performed a study similar to ours, where the clinical diagnosis is compared to the genetic diagnosis. Dostál *et al*. (2010) published a study in which they have genetically tested a large number of dogs of various breeds. Even though their allele frequency of the mutation in the *NHEJ1* gene in the Rough Collie was not as high as ours, it was remarkably high (0.797). This is, just like our results, indicative that it might be difficult to find genetically unaffected Rough Collies. Unfortunately, Dostál *et al.* (2010) have not included the clinical diagnosis of the dogs in their study, which is why no conclusion about the compliance between the clinical and genetic diagnosis can be drawn from their work.

The main question raised from the results we obtained is; why is our compliance so low? As discussed earlier, the penetrance of the genetic mutation is not believed to be 100% (Lowe *et al*. 2003), but it seems unlikely that this would explain the almost non-existing compliance (9%) in the clinically healthy dogs. Parker *et al*. (2007) consider it practical to use the presence of the deletion found in the *NHEJ1* gene as a genetic test for CRD. This clearly indicates that they believe the compliance between clinical and genetic diagnosis to be very good, if not perfect. They discuss that the test can be applied in breeding programs in order to reduce the prevalence of CEA. The genetic test is also discussed to be useful in situations where the clinical diagnosis is uncertain, such as where breeds that normally not associated with CRD are suspected to be affected, or in blue merle dogs where false diagnosis is possible due to pale eye colouring (Parker *et al*. 2007). On the contrary, if it is true that the penetrance is less than 100% and modifier loci might be involved in CRD, as discussed by Lowe *et al.* (2003), the genetic test is not completely reliable in these kinds of situations,

since genetically affected dogs might actually be clinically well. Even though Parker *et al*. (2007) do not discuss whether or not there might be other genes affecting the CRD phenotype than the mutation they have found in the *NHEJ1* gene, they do refer to the mutation as the primary CRD mutation. The fact that they call it the "primary" mutation, renders the reader in doubt of whether or not they believe that there are modifier genes involved in CRD or not, which is a relatively important detail when it comes to deciding how to practically implement the genetic test.

A reliable genetic test can in some situations be very useful for veterinarians, dog owners and dog breeders. In the case of CRD, a genetic test would make it possible to diagnose dogs that have passed the "golden age" of 10 weeks, with a higher certainty than possible today. This would be very helpful for breeders as well as veterinarians, as it might help them decide whether or not to use a certain individual in breeding. The fact that CRD is considered to be a recessive trait, further increases the use of a genetic test. If a breeder has a Collie that is clinically healthy, but is not assured of the CEA status of the parents or grandparents of the dog, a genetic test would in this situation allow the breeder to find out if the dog is a carrier of CRD or not. Nevertheless, since CRD is present in so many degrees of severity and is only one of the symptoms of CEA, it is naive to think that a genetic test could completely substitute a clinical examination.

As mentioned earlier, there are no breeding restrictions in Denmark when it comes to CRD, only recommendations. The genetic prevalence of the mutation for CRD in our study turned out to be exceptionally high, which, if representative for all of Denmark, would make it very difficult to find genetically healthy individuals to use for breeding. Because of the negative effects that inbreeding and decreasing the genetic pool within a breed can have, it seems that it is impossible to completely exclude dogs affected by CRD from breeding. It is not fully understood what it is that causes the severity of CRD to vary, but it cannot be ruled out that unknown genetic factors play a role. Because of this, it cannot be emphasized enough how important the clinical diagnosis is, and the importance of dogs with severe cases of CRD, and dogs with CRD in combination with other defects, should be recommended to be excluded from breeding. Hopefully, breeders in Denmark are well aware of the disease and try their

best to cooperate in order to reduce the problems as much as possible, without increasing the degree of inbreeding in the Rough Collie.

Conclusion

CRD is one of the main defects of CEA, and a lot of work has been done trying to find the candidate gene responsible for this defect. Currently, a genetic test for CRD is available, and the aim of this study was to investigate if there is compliance between clinical diagnosis and the genetic test for CRD, as well as trying to find if it seems possible to find genetically unaffected Rough Collies in Denmark. Our results show that none of the dogs included in this study were genetically unaffected. The compliance between the clinical and genetic diagnosis in our study turned out to be very poor, with only one of the clinically healthy individuals being a carrier, and the rest homozygous for the appointed causative mutation. As we do not find any satisfactory explanation for this lack of compliance, our conclusion is that, according to the results obtained in this study, the genetic test for CRD that exists today cannot be relied upon.

Perspectives

It would be desirable to conduct larger studies similar to the one presented here. Performing genetic testing on an extensive number of Collies, and other affected breeds, for the mutation in the *NHEJ1* gene, on dogs with a known clinical status, would give a better ground to conclude whether or not there is actual compliance between the genetic and the clinical diagnosis. If it turns out that the compliance in fact is poor, it would be advisable to thoroughly revise the genetic mapping of CRD. One might even have to consider starting from square one with an open mind, to explore the possibility of another gene than *NHEJ1* being the major gene of CRD, or the possibility of no single gene being responsible for the defect.

Without undermining the work done on the genetics of CRD, the authors of this essay find it more relevant to use future resources on trying to investigate the genetics of colobomas,

the more severe symptom of CEA. Since such a great percentage of the Collie population is affected by CRD, it is not possible to completely exclude dogs with CRD in their genes from breeding. Colobomas are not as prevalent as CRD, but cause blindness and defective vision to a much higher degree. Therefore, we find it more important to find a way of eradicating colobomas, which is why a genetic test for colobomas would be very usable for breeders of dogs affected by CEA. It has been speculated that colobomas are inherited as a dominant trait, and if that is true a genetic test could be considered to be of less importance. However, the clinical diagnosis is not a perfect tool, and it would be useful to have a genetic test as a complement.

In addition to this, we find it important to look over the currently existing breeding recommendation, and contemplate the possibility of introducing a breeding restriction for dogs with colobomas. It would also be preferable to introduce the requirement that Collies used for breeding must be ophthalmoscopically examined before the age of 10 weeks. It would be recommendable to examine the dogs at an adult age as well, since some of the secondary changes sometimes are not present in young dogs. These changes would hopefully decrease the prevalence of colobomas even further; reduce the problems with secondary changes, as well as minimizing the number of “go normals” used in breeding.

References

Barnett, K. C. (1979): Collie eye anomaly. *Journal small animal practice.* 20, 537-542.

Bedford, P. G. C. (1980): Incidence of collie eye anomaly. *Veterinary Record.* 95.

Bedford, P. G. C. (1982a): Collie eye anomaly in the border collie. *Veterinary Record.* 111, 34-35.

Bedford, P. G. C. (1982b): Collie eye anomaly in the United Kingdom. *Veterinary Record.* 111, 263-270.

Bedford. P**.** G. C. (1998): Collie eye anomaly in the Lancashire heeler*. Veterinary Record.* 143, 354-356.

Bjerkås, E. (1991): Collie eye anomaly in the rough collie in Norway. *Journal of Small Animal Practice.* 32**,** 89-92.

Collie.dk: *Sundhed,* [online]. Collie.dk. [quoted 3th of February 2014]. Available online: <URL: http://collie.dk/DK/Collien/Sundhed.aspx>.

Dansk Kennel Klub: *Øjensygdomme*, [online]. Dansk Kennel Klub, Solrød Strand. [quoted 5th of February 2014]. Available online: <URL: http:// www.dkk.dk/side.asp?ID=2444>.

Dansk Selskab for Veterinær Ophthalmologi (2013): *Avlsanbefalinger,* [online]. Dansk Selskab for Veterinær Ophthalmologi. [quoted 3th of February 2014]. Available online: <URL: http://dsvo.dk/avlsanbefalinger/>.

Donovan, E. F. & Wyman, M. (1965): Ocular fundus anomaly in the collie. *Journal of the American Veterinary Medical Association.* 147, 1465-1469.

Donovan, R.H., MacKenzie Freeman, H. & Schepens C.L. (1969): Anomaly of the Collie Eye. *Journal of the American Veterinary Medical Association*. 155, 872-875.

Dostál, J., Horák, P., Hrdlicová, A. & Stratil, A. (2010): Simplified PCR analysis of a mutation in the *NHEJ1* gene causing Collie eye anomaly in some dog breeds. *Czech J. Anim. Sci.* 55, 346-350.

Hartwell, L.H., Hood, L., Goldberg, M.L., Reynolds, A.E., Silver, L.M. & Veres, R.C. (2011): *Genetics from genes to genomes.* 4nd edition. McGraw Hill, New York.

König, H. E., & Liebich, H. G. (2009): *Veterinary Anatomy of Domestic Mammals*. 4th edition. Schattauer, Stuttgart.

Leppännen, M. & Saloniemi, H. (1998): Screening and controlling canine inherited ocular diseases in Finland: epidemiological, economical and health promotional aspect. *Veterinary Ophthalmology.* 1, 203-210.

Lowe, J. K., Kukekova, A. V., Kirkness, E. F., Langlois, M. C., Aguirre, G. D., Acland, G. M. & Ostrander, E. A. (2003): Linkage mapping of the primary disease locus for collie eye anomaly. *Genomics.* 82, 86-95.

Magrane, W. (1953): Congenital anomaly of the optic nerve in collies. *North American Veterinarian.* 34, 646.

McGavin., M. D. & Zachary., J. F. (2007): *Pathologic basis of veterinary disease.* 4th edition. Mosby Elsevier, Missouri.

Möller, I. (2014), personal communication. Ophthalmology specialist of veterinary medicine. Evidensia Djursjukhuset Helsingsborg. Bergavägen 3, 250 23 Helsingsborg. Tel. +46739-036324. email: ida.moeller@djursjukhus.com

Parker, H. G., Kukekkova, A. V., Akey, D. T., Goldstein, O., Kirkness, E. F., Baysac, K. C., Mosher, D. S., Aguirre, G. D., Acland, G. M. & Ostrander, E. A. (2007): Breed realtionships facilitate fine-mapping studier: A 7.8-kb deletion cosegregates with Collie eye anomaly across multiple dog breeds. *Genome Research.* 17, 1562-1571.

Roberts, S. R., Dellaporta, A. & Winter, F. C. (1966): The collie ectasia syndrome. Pathology of eyes of young and adult dogs. *American Journal of Ophthalmology.* 62, 728-752.

Sargan, D. R & Wallin-Håkanson (2001): Collie eye anomaly in the rough collie. *Journal of small animal practice.* 42. 204.

Stades, F. C. & Barnett, K. C.(1981): Collie Eye Anomaly in Collies in the Netherlands. *The veterinary quarterly.* 3, 66-73.

Strachan, T. & Read, A. (2011): *Human molecular genetics.* 4th edition. Garland Science, New York.

The editors of Encyclopaedia Britannica (1998): *Polymerase chain reaction,* [online]. Encyclopaedia Britannica, Inc. [quoted 15th of January 2014]. Available online: <URL: http://global.britannica.com/EBchecked/topic/468736/polymerase-chain-reaction (18122013)>.

The editors of Encyclopaedia Britannica (2013): *Human eye,* [online]. Encyclopaedia Britannica, Inc. [quoted 21th of January 2014]. Available online: <URL: http://global.britannica.com/EBchecked/topic/1688997/human-eye>.

Wallin-Håkanson, B., Wallin-Håkanson, N. & Hedhammar, Å. (2000a): Influence of selective breeding on the prevalence of chorioretinal dysplasia and coloboma in the rough collie in Sweden. *Journal of Small Animal Practice.* 41, 56-59.

Wallin-Håkanson, B., Wallin-Håkanson, N. & Hedhammar, Å. (2000b): Collie eye anomaly in the rough collie in Sweden: genetic transmission and influence on offspring vitality. *Journal of small animal practice.* 41, 254-258.

Walser-Reinhardt, L., Hässig, M. & Spiess, B. (2009): Collie Eye Anomaly in Switzerland. *Schweizer Archiv fuer Tierheilkunde.* 151, 597-603.

Yakely, W.L., Wyman, M., Donovan, E. F. & Fechheimer, N. s. (1968): Genetic Transmission of an Ocular Fundus Anomaly in Collies. *Journal of the American veterinary medical association.* 152, 457-461.

Yakely, W.L. (1972): Collie Eye Anomaly: Decreased Prevalence Through Selective Breeding. *Journal of the American veterinary medical association.* 161, 1103-1107.

Picture on front page taken from:
http://www.fanpop.com/clubs/roughcollie/images/10546262/title/pretty-collie-photo.
18th of February 2014

Appendix 1

Projektbeskrivelse, specialeprojekt om CEA

Baggrund:
CEA (Collie Eye Anomali) er et udbredt fænomen blandt collier over hele verden. I Danmark diagnosticeres CEA hos ca. 70 % af de collier, der øjenlyses. Ved diagnostikken skelnes der mellem CRD (Chorio-Retinal Dysplasi) og Colobom. CRD er en mangelfuld udvikling af årehinden, der giver blodforsyning til nethinden. CRD er til stede ved fødslen og øges ikke med alderen. Forandringerne kan maskeres af pigment, når nethinden skifter til "voksen" farve i 3 måneders alderen. Fænomenet kaldes "go-normal" og det sker hos ca. 30 % af de hundene der får diagnosticeret CRD som hvalpe. Hundene er dog langtfra blevet normale – forandringerne er bare blevet usynlige. Det bedste tidspunkt at diagnosticere CRD er i 6-10 ugers alderen. Tidligere inddeltes CRD i let, middel og svær på ECVO øjenattesterne, men det sker ikke mere.

Colobom er en mere alvorlig manifestation af CEA. Her mangler dele af senehinden og det kan give anledning til dannelse af "brok" hvor nethinde og blodkar presses ud. Der kan ses komplikationer i form af blødninger, nethindeløsning og blindhed.

Genetik:
CRD nedarves autosomal recessivt men der findes sandsynligvis andre gener, der har betydning for hvor alvorligt sygdommen udvikler sig. Genet for CRD sidder på kromosom 37 og syge hunde er homozygous for en 7.800 bp stor deletion i *NHEJ1* genet. Optigen, Laboklin og IDEXX Vetmedlab tilbyder en DNA-test for CEA. Testen er ikke patenteret.

Det nedenfor beskrevne studie opstilles med henblik på at øge kendskabet til sygdommen i den danske collie bestand og besvare følgende spørgsmål:

1) Er der overensstemmelse mellem den kliniske og den genetiske diagnose?
2) Kan der identificeres genetisk frie hunde i den danske population?

DNA-testen sættes op hos Merete Fredholm på LIFE og projektet gennemføres som et specialeprojekt. En gruppe udvalgte hunde, der er øjenlyst for CEA i 6-10 ugers alderen genotypes for deletionen i *NHEJ1* genet. Blandt de øjenlyste skal der være såvel fri som påviste og også gerne hunde med colobom. Klubben er behjælpelig med at udpege de relevante hunde – ca. 100 stk. Det er ønskværdigt med 50 hunde til analyserne og vi kan nok forvente ca. 50 % responsrate. Hvis det skal være muligt at beregne en valid allelfrekvens, skal hundene så vidt muligt være ubeslægtede.

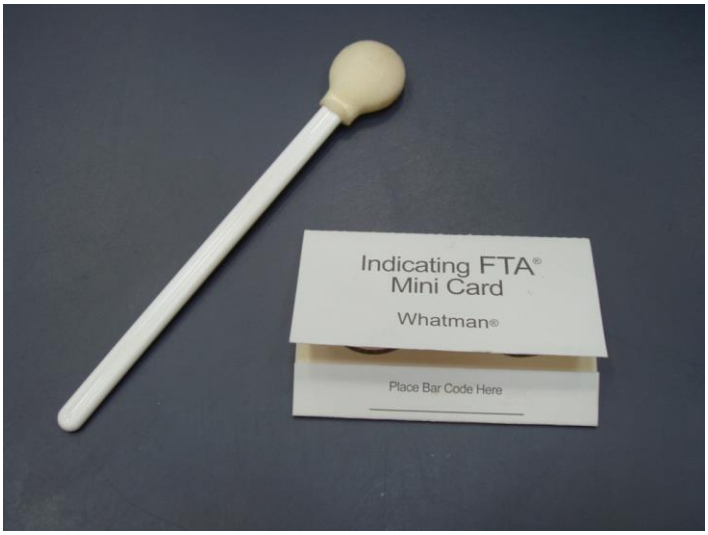
Ejerne til de udvalgte hunde får tilsendt et brev med prøveudtagningsudstyr i form af FTA kort samt en frankeret svarkuvert. Sammen med brevet vedlægges desuden en vejledning til prøve udtagningen, der allerede er fremstillet i forbindelse med et andet studie. På baggrund af genotypningen er det muligt at vurdere om der er overensstemmelse mellem klinisk CEA diagnose og homozygoti for deletionen. Det er desuden muligt at beregne en eksakt allelfrekvens.

Finansiering:
Collie Klubben dækker udgifter til svaber og porto (2x 100)
Der søges om midler fra DKK til dækning af laboratorieudgifter:

Opsætning af test	4.000 kr.
Oprensning af DNA og analyse ca. 100 kr./dyr	6.000 kr.
I alt	10.000 kr.

Appendix 2

VEJLEDNING
TIL OPSAMLING AF DNA
FRA MUNDSLIMHINDEN



1. Læs vejledningen inden du går i gang.

2. Tænk på følgende:

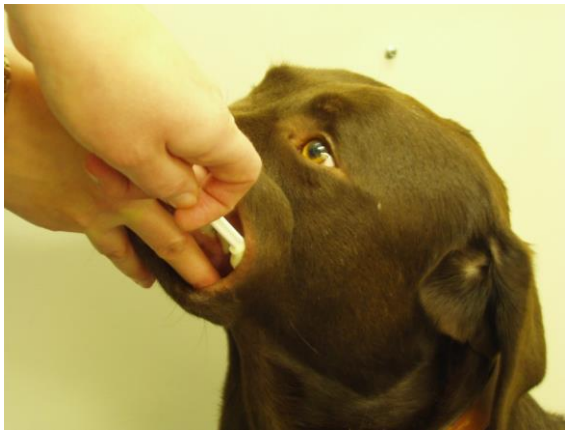
- ✓ Din hund skal ikke have spist eller drukket umiddelbart før prøvetagning.
- ✓ Undgå så vidt muligt at skumgummiet på prøvepinden kommer i kontakt med anden end hundens mundslimhinde og det lyserøde filtrerpapir på FTA-kortet.
- ✓ Når skumgummipuden har været i kontakt med FTA-kortet skal du ikke bruge den i hundens mund igen. Brug i stedet prøvepind nr. 2, hvis du er i tvivl om der er materiale nok.

3. Placer Jeres FTA-kort på en ren, tør og lige overflade.

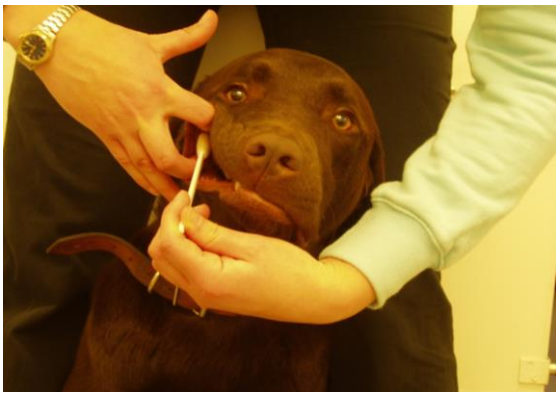
4. Skriv hundens komplette navn (inkl. kennelnavn) og stambogs-nummer på FTA-kortets forside.

5. Fjern plastikken fra prøvepindens pakning og åbn FTA-kortet så det lyserøde papir med de to sorte cirkler bliver synligt.

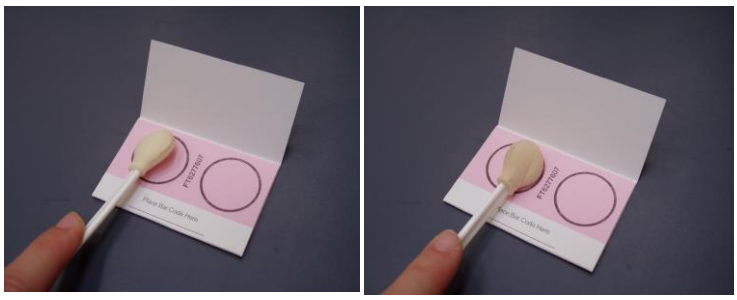
6. Adskil hundens over- og underlæbe ved hjælp af dine fingre. Få evt. en hjælper til at holde hunden hvis den er urolig. Hold prøvepinden i den anden hånd og før den ind på indersiden af kinden. Gnub skumgummiet forsigtigt mod indersiden af kinden i ca. 30 sekunder.



Gentag proceduren med den samme prøvepind i hundens anden kind. Til slut føres skumgummi-puden ned i bunden af mundhulen – evt. ind under tungen – for at opsuge så meget spyt som muligt.



7. Nu skal materialet overføres til filtrerpapiret. Tryk skumgummipuden mod papiret indenfor den ene sorte cirkel og vip puden fra side til side med en rullende bevægelse. **GNUB IKKE - papiret går nemt i stykker.** Vend skumgummipuden og overfør resten af materialet til den anden cirkel



8. Det lyserøde filtrerpapir skifter farve til hvidt når mundhuleskrabet overføres. Hvis dette ikke sker, er der ikke materiale nok, og du kan gentage proceduren med den vedlagte ekstra prøvepind.

9. Placer FTA-kortet som vist på billedet. Lad det lufttørre ved stuetemperatur i mindst 30 minutter.

10. Luk FTA-kortet, put det i den frankerede kuvert og send det med posten. Husk at vedlægge blanketten med dit navn og din adresse, så vi kan sende svaret til dig.

TUSIND TAK FOR HJÆLPEN!

Tak til Mette Lybeck Rueløkke, Hospital for Mindre Husdyr, for udlåning af labradoren Vitus

Appendix 3

Clinical Diagnosis of the dogs used in the study (CEA_001-CEA_045)

LAB ID	Hundens navn	Stambogsnummer	Kommentar
	Klok Collies Aviator	DK08725/2010	Hunde med colobom
	Lassies America James	DK12603/2010	Hunde med colobom
	Marselisborg's Braveheart	DK03696/2010	Hunde med colobom
CEA_010	Topperteam-s Go Get It	DK20786/2009	Hunde med colobom
	Daugaard Vonderfull Xander	DK03664/2009	Hunde med colobom
	Dyssemosens Black Bella	DK14101/2009	colobom + nethindeløsning
	Alislina's Vilo Tjørn Aiko	DK12394/2008	Hunde med colobom
	Uglebjerg Big Mac	DK15277/2008	Hunde med colobom
	Kiva's Eden White Oleander	DK12732/2008	Hunde med colobom
CEA_030	Bijanti Collie's Dreamy Night	DK00519/2009	Hunde med colobom
CEA_003	Dyssemosen's Black Atilla	DK13193/2008	Hunde med colobom
	Si Si Wizard Of Bluebell	DK16788/2007	colobom + nethindeløsning
	Shep's Feel The Power	DK02017/2007	Hunde med colobom
	Be Eksklusive Midnight Dream	DK08379/2007	Hunde med colobom
-kom retur	Rincols Eo Ipso	DK02394/2007	Hunde med colobom
CEA_020	Daugaard Vonderfull Dravo	DK03873/2012	Hunde klinisk fri
CEA_029	Pahlex Dream Baby	DK19458/2011	Hunde klinisk fri
CEA_004	Kiva's Eden Fortress Of Freedom	DK00518/2012	Hunde klinisk fri
CEA_033	Almais Placido Domingo	DK07749/2011	Hunde klinisk fri
- DØD	Belrosa My Soulmate	DK04748/2011	Hunde klinisk fri
CEA_009	Be Exclusives Endless Love	DK08227/2011	Hunde klinisk fri
	Daugaard Wonderfull Amy	DK02172/2011	Hunde klinisk fri
CEA_007	Topperteam Hot Summer Love	DK17385/2010	Hunde klinisk fri
CEA_021	Trebricha's Masser Af Succes	DK10582/2010	Hunde klinisk fri
	Lapinette's Chelsa Brigde	DK10205/2010	Hunde klinisk fri
	Alisina's Canis Major Bazil	DK15862/2010	Hunde klinisk fri
CEA_041	Topperteam's In Your Dreams	DK20789/2009	Hunde klinisk fri
	Sungrace Reasonable Rumba	DK08441/2009	Hunde klinisk fri
CEA_040	Shep's Kiss N'Tell	DK06536/2009	Hunde klinisk fri
CEA_025	Daugaard Vonderfull Blue Yuvel	DK00696/2010	Hunde klinisk fri
	Sheps's Love To Be Loved	DK22939/2009	Hunde klinisk fri
	Lapinette's Back In Black	DK22142/2008	Hunde klinisk fri

CEA_031	Pahlex Dollar N Dime	DK22104/2005	Hunde klinisk fri
CEA_023	Klok Collies Calandar Girl	DK02626/2012	Hunde med med CRD
CEA_002	Bjianti Collies Chic Black Krystal	DK07041/2012	Hunde med med CRD
CEA_039	Vielsted's Happy	DK05081/2012	Hunde med med CRD
CEA_034	Lassies Sweet American Kiwi	DK04049/2012	Hunde med med CRD
	Uglebjerg Fashion Star	DK04580/2012	Hunde med med CRD
	Lapinette's Disco Diva	DK13383/2011	Hunde med med CRD
	Treasure Collies Beyond Limits	DK07145/2011	Hunde med med CRD
CEA_005	Swinger Hot'N Tot	DK10143/2011	Hunde med med CRD
	Topperteam's Moonlight Affair	DK10243/2011	Hunde med med CRD
CEA_044	Si-Si's Snow Angel	DK01136/2011	Hunde med med CRD
CEA_036	Rollington Time Will Tell	DK08012/2011	Hunde med med CRD
CEA_022	Sønderby's She's Got The Look	DK18772//2010	Hunde med med CRD
	Lassie's American Idol	DK12594/2010	Hunde med med CRD
	Lapinette's Chick CoreA	DK10208/2010	Hunde med med CRD
	Kiva's Eden Above Jacks And Kings	DK08911/2010	Hunde med med CRD
CEA_017	Ebony Moor's Adorable Alba	DK16075/2010	Hunde med med CRD
CEA_012	Be'Exclusive Spirit Of Legends	DK05146/2010	Hunde med med CRD
	Alisina's Leo Minor Balder	DK15863/2010	Hunde med med CRD
CEA_026	Klok Collies Against The Wind	DK08722/2010	Hunde med med CRD
	Lassies American Haino	DK05408/2010	Hunde med med CRD
	Sungrace Satisfy Sally	DK10591/2010	Hunde med med CRD
	Vielsted's Elliot	DK01104/2010	Hunde med med CRD
	Daugaard Vonderfull Xhara	DK03671/2009	Hunde med med CRD
CEA_019	Kiva's Eden Xtra Black prince	DK03043/2009	Hunde med med CRD
CEA_001	Aynsley Sugar N* Gold	DK11117/2009	Hunde med med CRD
	Calina Collies Almost An Angel	DK20913/2009	Hunde med med CRD
	Heitmann Effie Golden Tresure	DK10319/2009	Hunde med med CRD
	Topperteam With Love From Germany	DK20787/2009	Hunde med med CRD
CEA_038	Treasure Collie's Angel Eyes	DK12066/2009	Hunde med med CRD
CEA_042	Si-Si's Prima La Perla	DK12308/2009	Hunde med med CRD
	Shep's Garcia Golpe De Maestro		Hunde med med CRD
CEA_024	Sungrace Reasonable Rosa	DK08440/2009	Hunde med med CRD
CEA_032	Almais Lady Snowberry	DK01870/2010	Hunde med med CRD
CEA_045	Shep's Like A Dram	DK22934/2009	Hunde med med CRD
CEA_027	Uglebjerg Win Win Situation	DK23291/2008	Hunde med med CRD
CEA_037	Lassie's Glowing gaby	DK19163/2008	Hunde med med CRD
	Lassie's American Fellow	DK18486/2008	Hunde med med CRD
	Almais Cafe Solo /	DK16910/2008	Hunde med med CRD
	Alisina's Kastanjeblomst Anett	DK12393/2008	Hunde med med CRD

CEA_043	Si-Si's Knight Of Blue Heaven	DK08879/2008	Hunde med med CRD
CEA_013	Daugaard Vonderfull Vicki	DK09691/2008	Hunde med med CRD
	Uglebjerg Berlusconi Blue	DK15272/2008	Hunde med med CRD
- udstationeret	Kiva's Eden Enchanthing Black Night	DK14458/2008	Hunde med med CRD
CEA_015	Bijanti Collie's Amita Beatty	DK05954/2008	Hunde med med CRD
CEA_018	Belrosa Mystery Woman	DK02927/2008	Hunde med med CRD
CEA_006	Swinger Hot N' Spicy	DK06319/2008	Hunde med med CRD
	Lassie's American Explorer	DK03007/2008	Hunde med med CRD
- kom retur	Bijanti Collie's Aika A Dream		Hunde med med CRD
CEA_028	Pahlex Good Night Irene	DK01732/2008	Hunde med med CRD
CEA_011	Be' Exclusive's Single Attraktion	DK05846/2007	Hunde med med CRD
CEA_008	Lapinette's Prima Donna	DK21244/2007	Hunde med med CRD
	Topperteam's Guardian Angel	DK16993/2007	Hunde med med CRD
	Shep's Feel's Like heaven	DK02016/2007	Hunde med med CRD
CEA_016	Rockley's Kindly Kira Of Hope	DK03936/2007	Hunde med med CRD
CEA_035	Rollington Perhabs Perhabs Perhabs	DK09835/2007	Hunde med med CRD
	Si'Si's Wizard Of Bluebell	DK16788/2007	Hunde med med CRD
CEA_014	Sungrace Quality Quicki	DK15908/2007	Hunde med med CRD

Appendix 4

QIAamp DNA Investigator Handbook 12/2007

Protocol: Isolation of Total DNA from FTA and Guthrie Cards. This protocol is for isolation of total (genomic and mitochondrial) DNA from whole blood, saliva, or buccal cells dried and immobilized on FTA cards, Guthrie cards, or similar collection devices.

Important point before starting

■ Perform all centrifugation steps at room temperature (15–25°C).

Things to do before starting

■ Equilibrate Buffer ATE or distilled water for elution to room temperature (15–25°C).
■ If Buffer AL or Buffer ATL contains precipitates, dissolve by heating to 70°C with gentle agitation.
■ Set a thermomixer or heated orbital incubator to 56°C for use in step 4 and (optional) step 16, and a second thermomixer or heated orbital incubator to 70°C for use in step 7. If thermomixers or heated orbital incubators are not available, heating blocks or water baths can be used instead.
■ Ensure that Buffers AW1 and AW2 have been prepared according to the instructions on page 12.
■ Optional: If processing very small amounts of starting material, add carrier RNA dissolved in Buffer ATE to Buffer AL according to the instructions on page 13.

Procedure

1. Cut 3 mm (1/8 inch) diameter punches from a dried spot with a single-hole paper punch. Place up to 3 card punches into a 1.5 ml microcentrifuge tube (not provided).
2. Add 280 µl Buffer ATL.
3. Add 20 µl proteinase K and mix thoroughly by vortexing.
4. Place the 1.5 ml tube in a thermomixer or heated orbital incubator, and incubate at 56°C with shaking at 900 rpm for 1 h. If using a heating block or water bath, vortex the tube for 10 s every 10 min to improve lysis.
5. Briefly centrifuge the 1.5 ml tube to remove drops from the inside of the lid.
6. Add 300 µl Buffer AL, close the lid, and mix by pulse-vortexing for 10 s. To ensure efficient lysis, it is essential that the sample and Buffer AL are thoroughly mixed to yield a homogeneous solution. A white precipitate may form when Buffer AL is added to Buffer ATL. The precipitate does not interfere with the QIAamp procedure and will dissolve during the heat incubation in step 7.
Note: If processing only 1 blood card punch with a diameter of 3 mm or less, we recommend adding carrier RNA to Buffer AL (see page 13). Note that carrier RNA does not dissolve in Buffer AL. It must first be dissolved in Buffer ATE and then added to Buffer AL.
7. Place the 1.5 ml tube in a thermomixer or heated orbital incubator, and incubate at 70°C with shaking at 900 rpm for 10 min. If using a heating block or water bath, vortex the tube for 10 s every 3 min.
8. Briefly centrifuge the 1.5 ml tube to remove drops from the inside of the lid.
9. Add 150 µl ethanol (96–100%), close the lid, and mix thoroughly by pulse- vortexing for 15 s. To ensure efficient binding in step 11, it is essential that the sample and ethanol are thoroughly mixed to yield a homogeneous solution.
10. Briefly centrifuge the 1.5 ml tube to remove drops from the inside of the lid.
11. Carefully transfer the entire lysate from step 10 to the QIAamp MinElute column (in a 2 ml collection tube) without wetting the rim, close the lid, and centrifuge at 6000 x g (8000 rpm) for 1 min. Place the QIAamp

MinElute column in a clean 2 ml collection tube, and discard the collection tube containing the flow-through. If the lysate has not completely passed through the membrane after centrifugation, centrifuge again at a higher speed until the QIAamp MinElute column is empty.

12. Carefully open the QIAamp MinElute column and add 500 µl Buffer AW1 without wetting the rim. Close the lid and centrifuge at 6000 x g (8000 rpm) for 1 min. Place the QIAamp MinElute column in a clean 2 ml collection tube, and discard the collection tube containing the flow-through.

13. Carefully open the QIAamp MinElute column and add 700 µl Buffer AW2 without wetting the rim. Close the lid and centrifuge at 6000 x g (8000 rpm) for 1 min. Place the QIAamp MinElute column in a clean 2 ml collection tube, and discard the collection tube containing the flow-through. Contact between the QIAamp MinElute column and the flow-through should be avoided. Some centrifuge rotors may vibrate upon deceleration, resulting in the flow-through, which contains ethanol, coming into contact with the QIAamp MinElute column. Take care when removing the QIAamp MinElute column and collection tube from the rotor, so that flow-through does not come into contact with the QIAamp MinElute column.

14. Carefully open the QIAamp MinElute column, and add 700 µl of ethanol (96–100%) without wetting the rim. Close the cap and centrifuge at 6000 x g (8000 rpm) for 1 min. Place the QIAamp MinElute column in a clean 2 ml collection tube, and discard the collection tube containing the flow-through.

15. Centrifuge at full speed (20,000 x g; 14,000 rpm) for 3 min to dry the membrane completely.
This step is necessary, since ethanol carryover into the eluate may interfere with some downstream applications.

16. Place the QIAamp MinElute column in a clean 1.5 ml microcentrifuge tube (not provided), and discard the collection tube containing the flow-through. Carefully open the lid of the QIAamp MinElute column, and incubate at room temperature (15–25°C) for 10 min or at 56°C for 3 min.

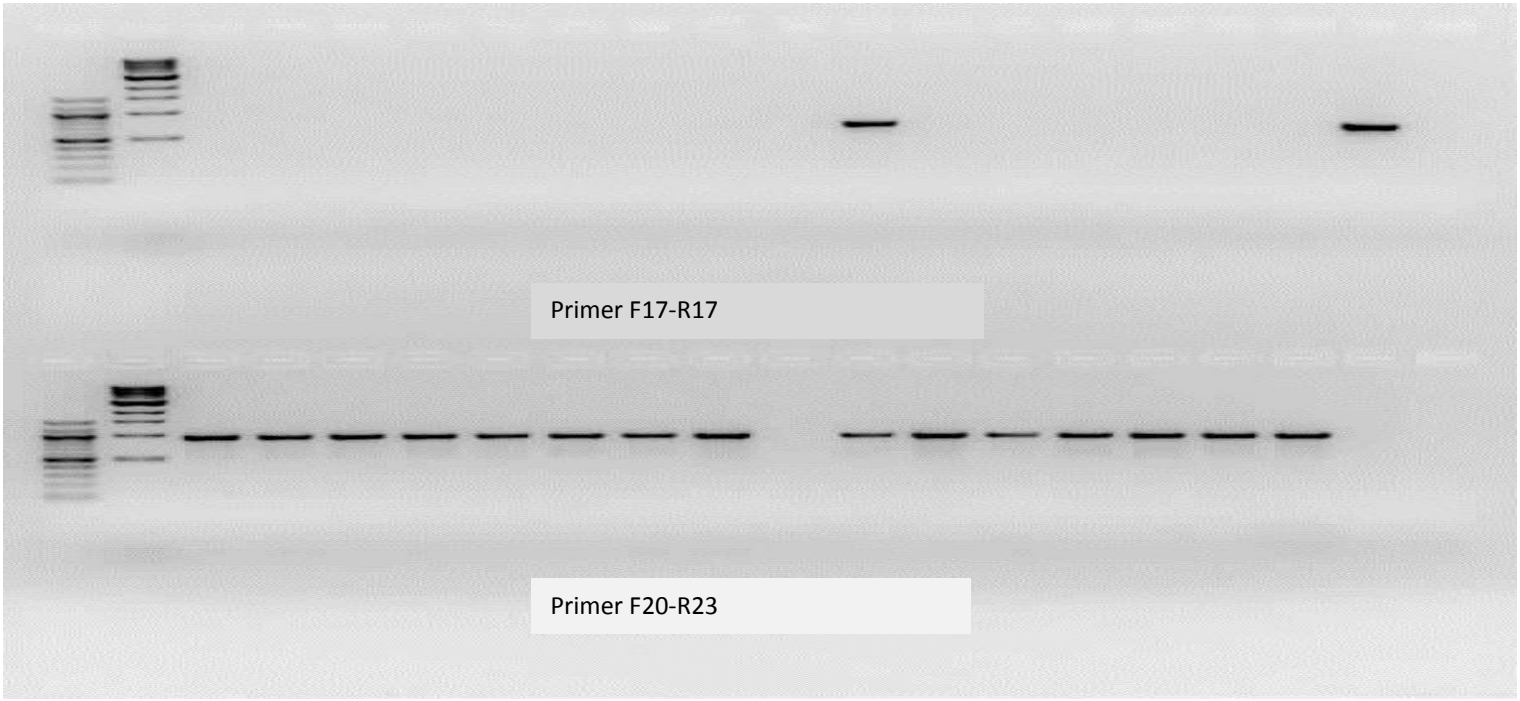
17. Apply 20–100 µl Buffer ATE or distilled water to the center of the membrane.
Important: Ensure that Buffer ATE or distilled water is equilibrated to room temperature (15–25°C). If using small elution volumes (<50 µl), dispense Buffer ATE or distilled water onto the center of the membrane to ensure complete elution of bound DNA. QIAamp MinElute columns provide flexibility in the choice of elution volume. Choose a volume according to the requirements of the downstream application. Elution with small volumes increases the final DNA concentration in the eluate significantly, but reduces the overall DNA yield. Remember that the volume of eluate will be up to 5 µl less than the volume of elution solution applied to the column.

18. Close the lid and incubate at room temperature (15–25°C) for 1 min. Centrifuge at full speed (20,000 x g; 14,000 rpm) for 1 min. Incubating the QIAamp MinElute column loaded with Buffer ATE or water for 5 min at room temperature before centrifugation generally increases DNA yield.

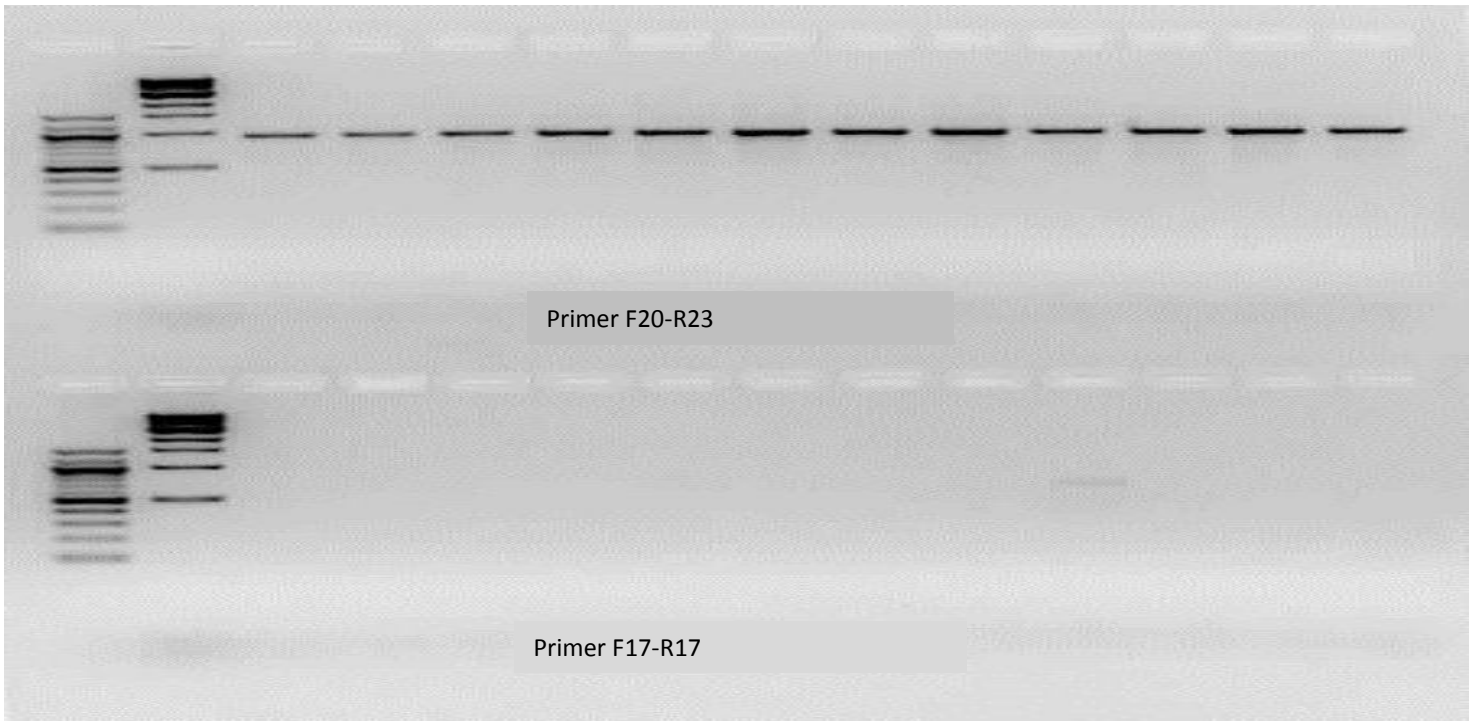
Appendix 5

Electrophoretogram demonstrating PCR results

100 bp	1 kbp	CEA 012	CEA 013	CEA 014	CEA 015	CEA 016	CEA 017	CEA 018	CEA 019	CEA 020	CEA 021	CEA 022	CEA 023	CEA 024	CEA 025	CEA 026	CEA 027	DDG 118	H_2O



100 bp	1 kbp	CEA 028	CEA 029	CEA 030	CEA 031	CEA 032	CEA 033	CEA 034	CEA 035	CEA 036	CEA 037	CEA 038	CEA 039



100 bp	1 kbp	CEA 040	CEA 041	CEA 042	CEA 043	CEA 044	CEA 045	H_2O	H_2O	CEA 020	CEA 021	CEA 001	DDG 118