UNIVERSITY OF COPENHAGEN
FACULTY OF HEALTH AND MEDICAL SCIENCES
Department of Veterinary Clinical and Animal Sciences
Division of Genetics, Bioinformatics and Breeding

Master's thesis

Collie Eye Anomaly

Prevalence of the *NHEJ1* gene mutation in the Danish Shetland Sheepdog and the effects on vision in dogs diagnosed with collie eye anomaly.

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Preface

This Master's thesis finalizes my degree in veterinary medicine. It was conducted in the period from August 2014 to February 2015 at the division of Genetics, Bioinformatics and Breeding, Department of Veterinary Clinical and Animal Sciences, Faculty of Health and Medical Services, Copenhagen University.

The purpose of this project is to provide veterinarians, breeders and breeding associations with knowledge of the prevalence of CEA in Danish Shetland Sheepdogs, and also to further investigate the compliance between the clinical diagnosis of CEA and the genetic test available. Moreover, it intends to give insight into the visional effects on dogs affected with CEA.

The present study is a successor to a previous Master's thesis done by Marie Jönsson and Marie Annie Söderlund, 2014. In their study, an investigation of the allele frequency of the *NHEJ1* gene mutation was conducted in the Danish Rough Collie population and an evaluation of the compliance between the clinical diagnosis and the DNA-test was made.

Tølløse, Denmark, February 13th 2015

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Rikke Crone Larsen

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Furthermore I give a very special thanks to my academic advisor, Professor Merete Fredholm, for being my guide and counsel throughout the process. Her willingness to share information with me from her tremendous knowledgebase and her ability of always making me feel able, has been completely invaluable.

Last but no certainly not least, I would like to thank my family and friends who have given me a tremendous amount support throughout the process of writing this thesis and in pursuing my lifelong dream of becoming a veterinarian.

Abstract

Background: Collie Eye Anomaly is a congenital, inherited ocular disorder with very variable phenotypic expression. CEA has primarily been demonstrated in herding breeds such as the Shetland Sheepdog. Reports on visional effects ranging from no obvious signs of affliction to complete blindness have been made, but scientific studies on the subject are utterly sparse.

Recently, a commercially available genetic test for CRD has been developed. The DNA-test tests for a 7,8 bp deletion in the *NHEJ1* gene on canine chromosome 37. The compliance between the genetic test and the clinical diagnosis of CRD have previously been investigated in Danish Rough Collies and found to be poor. Further studies of the association are therefore indicated.

Objective: The aims of the study were to further investigate the compliance between the genetic test and the clinical diagnosis of CRD, by testing another known-affected breed, the Shetland Sheepdog. Also the prevalence of the mutation in the breed were sought to be established. Moreover, insight into the visional effects of the anomaly was desired.

Methods: The study was conducted partially as a case-control study and partially by use of a questionnaire. The case-control study population consisted of unrelated, clinically affected- and unaffected Shetland Sheepdogs which were eye examined before 12 weeks of age. The study population for the questionnaire was owners of dogs participating in the current case-control study, and those who participated in the previous study in Rough Collies.

DNA samples from the buccal mucosa of selected dogs were collected on FTA cards and subsequently genotyped. Genotyping was done by the use of a two-step PCR program.

Questionnaires were distributed either via mail or via letter. Responses were collected and analyzed.

Results:

A high degree of compliance between the clinical diagnosis and the genetic test was found. Inconsistencies between the two diagnosis were only found in 5/56 cases. All of dogs were blue merle. The allele frequency of the *NHEJ1* gene mutation was found to be 0,58.

Based on the owners' perceptions, vision in affected as well as unaffected dogs seems to be normal. The only report of inferior vision was in a dog with bilateral coloboma.

Conclusion: The mutation allele frequency found was 0,58. A high degree of compliance between the genetic test and the clinical diagnosis in the Shetland Sheepdogs was found. However, uncertainties of the presumed mode of inheritance of CRD were presented and therefore the use of the genetic test as the sole diagnostic tool is not advised.

Based on owners observations, vision in most affected dogs were found to be normal. Data on the subject of vision was found to be sparse and therefore scientific studies are needed to shed further light on the visional effects of the anomaly.

Resumé

Baggrund: Collie Eye Anomaly er en medfødt, arvelig lidelse med et meget variabelt fænotypisk udtryk. CEA er primært blevet fundet i hyrdehunde racer som for eksempel Shetland Sheepdog. Rapporteringer af de synsmæssige påvirkninger rangerer fra ingen tydeligelige tegn på påvirkning til fuldstændig blindhed, men videnskabelige studier på området er meget sparsomme.

Fornyligt er der blevet udviklet en kommerciel tilgængelig genetisk test for CRD. DNA-testen tester for en 7,8 bp deletion i *NHEJ1* genet på hundes kromosom 37. Overenstemmelse mellem den genetiske test og den kliniske diagnose ad CRD er tidligere blevet undersøgt i danske langhårrede collies og fundet til at være dårlig. Yderligere studier af sammenhængen er derfor indikeret.

Formål: Formålet med studiet var at undersøge overensstemmelsen mellem den genetiske- og den kliniske diagnose af CRD yderligere, ved at teste en anden kendt afficeret race, den Shetlandske Sheepdog. Prevalensen af mutationen i racen søgtes også at blive slået fast. Derudover ønskes der indsigt i de synsmæssige påvirkninger af anomalien.

Metode: Studiet blev udført delvist som et case-kontrol studie og delvist ved brug af et spørgeskema. Case-kontrol studiepopulationen bestod af ubeslægtede klinisk afficerede- og uafficerede Shetland Sheepdogs der var blevet øjenundersøgt inden 12 ugers alderen. Studiepopulationen til spørgeskemaet var ejere af hunde der deltog i det aktuelle studie, og også de der deltog i det forudgående studie i langhårede collies.

DNA prøver fra mundslimhinden af udvalgte hunde blev indsamlet på FTA kort og efterfølgende genotypet. Genotypningen blev udført ved brug af et to-trins PCR program. Spørgeskemaerne blev distribueret enten via mail eller via brev. Svarene blev indsamlet og analyseret.

Resultater: Der blev fundet en høj grad af overensstemmelse mellem den kliniske diagnose og den genetiske test. Uoverensstemmelser mellem de to diagnoser blev kun fundet i 5/56 prøver. Alle disse hunde var blue merle. Allelfrekvensen af *NHEJ1* gen mutationen blev fundet til at være 0,58. Baseret på ejernes opfattelser synes synet at være normalt både i påvirkede og ikke påvirkede hunde. Den eneste rapport om nedsat syn var i en hund med bilateralt colobom.

Konklusion: Allelfrekvensen af mutationen var 0,58. En høj grad af overensstemmelse mellem den genetiske test og den kliniske diagnose blev fundet i Shetland Sheepdog hundene. Midlertidigt er der fremlagt usikkerhed vedrørende den formodede arvegang af CRD, og på denne baggrund bør den genetiske test ikke bruges som eneste diagnostiske redskab.

Baseret på ejernes observationer blev synet fundet til at være normalt i de fleste påvirkede hunde. Data på emnet syn blev fundet til at være meget sparsomme og derfor er videnskabelige studier nødvendige for at kaste yderligere lys over de synsmæssige effekter af anomalien.

Contents

ABBREVIATIONS **2**
INTRODUCTION **3**
1 Aims **4**
2 Methods and limitations **5**
LITERATURE REVIEW **6**
3 The Eye **6**
3.1 Sclera 6
3.2 Choroidea 6
3.3 Retina 8
3.4 Optic disc and Optic nerve 10
4 CEA **11**
4.1 Clinical pathology of CEA 11
4.1.1 Chorioretinal dysplasia(CRD) / Choriodal hypoplasia (CH) 11
4.1.2 Coloboma 13
4.1.3 Tortuous retinal vessels 14
4.1.4 Secondary Changes 15
4.2 Vision in CEA affected dogs 15
4.3 Embryologic development of CEA 16
4.4 Clinical diagnosis 17
5 Genetics **18**
5.1 Genetics of collie eye anomaly 19
5.2 Genetic mapping of CRD 21
5.3 Genotyping by the use of polymerase chain reaction 24
6 Breeding restrictions and recommendations 26
CASE CONTROL STUDY/QUESTIONNAIRE **27**
7 Materials **27**
7.1 Genotyping of Shetland Sheepdogs 27
7.2 Questionnaire 28
8 Methods **28**
8.1 Genotyping of Shetland Sheepdogs 28
8.2 Questionnaire 30
9 Results **31**
9.1 Genotyping 31

9.2 Questionnaires 32
DISCUSSION 34
CONCLUSION 39
PERSPECTIVES 41
REFERENCES 43
APPENDIX I
Appendix A– Buccal mucosal DNA sample collection guide I
Appendix B – Whatman® FTA card manual V
Appendix C - Electrophoretograms VII
Appendix D – Results of genotyping XII

ABBREVIATIONS

ACVO: American College of Veterinary Opthalmologists

BAC: Bacterial artificial chromosome

Bp: Base pair

CEA: Collie Eye Anomaly

CH: Choroidal hypoplasia

cM: Centimorgan

CRD: Chorioretinal dysplasia

DKK: Danish Kennel Club

dNTP: Deoxynucleotide triphosphates

ECVO: European College of Veterinary Ophthalmologists

ED: Elbow dysplasia

FTA: Flinders Technical Associates

HD: Hip dysplasia

LOD: Logarithm of the odds

$MgCl_2$: Magnesium chloride

NHEJ1: Non-homologous end-joining factor 1

OCT: Optical coherence tomography

PCR: Polymerase chain reaction

PHPV: Persistent hyperplastic primary vitreous

PHTVL: Persistent hyperplastic tunica vasculosa lentis

PRA: Progressive retinal atrophy

RPE: Retinal pigment epithelium

SNP: Single Nucleotide Polymorphism

Taq: *Thermus Aquaticus*

UV: Ultra violet

INTRODUCTION

In 1953, Magrane was the first to describe cases of Collies which presented with similar, presumably inherited ocular anomalies located to the posterior segment of the eye (Magrane, 1953). These anomalies were later found in many members of the collie breed and lesions were therefore collectively named, Collie Eye Anomaly (CEA). Subsequently the anomaly has been documented in other breeds both of herding and non-herding origin (Barnett and Stades, 1979; Bedford, 1982b; Bedford, 1998; Munyard, et al., 2007; Mizukami, et al., 2012).

CEA have been widely recognized in Shetland Sheepdogs with prevalences of 48,3% in the Netherlands (Barnett and Stades, 1979), 72% in the UK (Bedford, 1982a) and 15,1 % in Switzerland (Walser-Reinhardt, et al., 2009).

Clinically, the two major lesions associated with CEA are chorioretinal dysplasia (CRD) and coloboma. CRD, the primary phenotype, affects the retina and choroid causing pigment depleted "pale areas". Coloboma is characterized by excavations within or adjacent to the optic disc. Lesions are commonly present bilaterally but often of asymmetrical distribution. The clinical expression of the disease is very variable among individuals as is the degree of visual affliction (Barnett, 1979).

In order to get an accurate clinical diagnosis of CRD, ophthalmological examination within the first 10 weeks of the puppy' life it is paramount. After this period the retina changes color and minor CRD changes can be masked, the so called "go-normal" effect (Bedford, 1982a; Bjerkås, 1991).

Scientific discussions of the mode of inheritance of the CRD and coloboma have taken place throughout the years. Some authors believe that CRD is a trait with simple autosomale recessive inheritance (Lowe, et al., 2003; Parker, et al., 2007) whereas other believe it has a polygentic autosomal mode of inheritance (Wallin-Håkanson, et al., 2000b). Coloboma has been suggested to be inherited with CRD (Yakely, et al., 1968) or as a separate trait (Donovan, et al., 1969; Wallin-Håkanson, et al., 2000a)

In recent years, CRD affected dog have been found to share a 7,8bp deletion within intron 4 on canine chromosome 37 (Parker, et al., 2007). This finding has led to the development of a commercially available genetic test for CRD.

In 2014, a Danish master's thesis examined the compliance between the genetic test and the clinical diagnosis in Rough Collies and interestingly found a poor compliance (Jönsson and Söderlund, 2014). Similar studies in other affected breeds such as the Shetland Sheepdog are therefore indicated in order to investigate the relationship more closely. These studies offer the possibility of a simultaneous investigation of the genotype- and allele frequencies in the breed.

Exceedingly sparse amounts of data exist on the actual visual effects of the different degrees of the anomaly. Generally, severe visional impairment or blindness has been associated with retinal detachment or intraocular hemorrhage. Minor changes have been reported not to affect vision (Barnett, 1979; Bedford, 1982a). Interestingly, it has been reported that vision in affected eyes may be much less than the owner expects (Blogg, 1970) and that vision in CRD affected eye are in some way compromised although not clinically detectable (Donovan and Wyman, 1965)

On this basis, further knowledge of the prevalence, the diagnostic compliance and the effects of vision would bring additional insight into the complexity of CEA and thereby be usable tools for veterinarians, breeding associations, breeders and dog owners.

1 Aims

The aims of this project are:

1) To further investigate the compliance between the clinical diagnosis of CRD and the presumed causative mutation in the *NHEJ1* gene.

2) To investigate the prevalence of the *NHEJ1* gene mutation in the Danish Shetland Sheepdog population.

2) To investigate the visional effects of CEA.

2 Methods and limitations

This content of this thesis was substantiated by peer reviewed articles, a number of textbooks and some webpages. All articles were found via Pubmed or Medline in the period September 2014 to February 2015. Textbooks and webpages were selected based on relevance.

The study was limited to a period of 26 weeks, corresponding to 30 ECTS points. This short period of time has limited the extent of the study. If we had had more time, more dogs should have been included to strengthen the statistical validity.

The thesis consists of three parts; A literature review, a case-control study and a questionnaire survey.

The literature review begins with descriptions of ocular anatomy and physiology relevant to CEA. Hereafter follows a detailed section on the clinical pathology of CEA, the embryologic development and the clinical diagnosis. Next, a genetics section where current and past studies of CEA genetics is presented and finally a section on the breeding restrictions and recommendation of CEA.

The case-control study investigated the prevalence of the genetic mutation of the *NHEJ1*gene in a population of 56 Shetland Sheepdogs. These dogs were selected by a member of the Health Committee from the Danish Shetland Sheepdog Club. All dogs were eye examined between 5-12 weeks of age and they were unrelated on parental level.

The questionnaire survey was sent to the owners of the 45 Rough Collies who participated in the previous CEA study. These dogs were selected on basis of the same criteria as those of our case-control study.
Also 30 Shetland Sheepdogs owners, those who wanted to receive the DNA-test results of their dogs, received the questionnaire. The questions in the questionnaire were formed by the author in collaboration with an ophthalmological specialist and my thesis supervisor.

LITERATURE REVIEW

3 The Eye

The eye of mammals consists largely of three chambers and three tunics. From rostral to caudal the chambers are; the anterior chamber between the cornea and iris; the posterior chamber between the iris, ciliary body and lens; and the vitreous chamber behind the lens, surrounded by the retina (Liebich and König, 2007) .

The outermost of the three tunics is a fibrous layer composed of dense collagen. The anterior part consists of the transparent cornea and the posterior part is the whitish-colored sclera.

The middle tunic contains the choroid, the ciliary body and iris. The innermost tunic includes the visual and non-visual parts of the retina (Liebich and König, 2007).

In the following sections, the ocular structures relevant to the CEA syndrome will be described in more detail. These structures are found in the sclera, the ocular fundus and the optic disc/nerve.

3.1 Sclera

The sclera consists largely of a dense network of collagen with interlaced elastic fibres. The eleasticity of the sclera help they eyeball to maintain its shape and size by resisting the intraocular pressure. Another important function of the sclera is found at the corneoscleral junction in the scleral venous plexus. This structure regulates the ocular pressure by draining the aqueous humor. It is normal for the sclera to varry in thickness, being thinnest near to the equator and gaining thickness towards the posterior pole of the eye. The coloration of the sclera also varry, appearing blue when the sclera is thin. The point of which the optic nerve passes through the sclera is known as the lamina cribosa (Samuelson, 2007; Liebich and König, 2007).

3.2 Choroidea

The coroid is the posterior part of the middle tunic of the eye called the uvea. It is a highly vascularized layer which primary function is to supply the outer layers of the retina with nutrients and oxygen (Samuelson, 2007).

Morphologically the choroid can be divided into four layers (Samuelson, 2007).

Externally to internally:

- The suprachoroidea
- Large vessel layer
- Medium vessel layer and tapetum cellulosum
- The choriocapillaris

The suprachoroidea makes the transition between the sclera and the rest of the choroid. It consists of elastic connective tissue which contains large amounts of pigment.

Adjecent to the suprachoidea lies the large vessel layer containing a vascular plexus sourrounded by loose connective tissue. The plexus is made up by large veins and a smaller number of arteries. The arteries provides the outer part of the retina with nutrients, and may also serve as a "cooler" by removing heat produced by absorption of light (Samuelson, 2007)

Internally to the large vessel layer is a layer of medium-sized vessels. The vessels of this layer serve as a interconnection between the large vessel layer and the choriocapillary layer (Samuelson, 2007). The choriocapillaries is the final branching of the arteries, which supply the outer retina with nutrition.

Large numbers of pigmentcontaining melanocytes are found within the medium-sized vessel layer. In this same layer, in the area of the superior fundus, lies an important structure called the tapetum lucidum (Samuelson, 2007).

In carnivores, the tapetum is cellular in structure and it is therefore often refered to as the tapetum cellulosum. The cells of this layer are specific polyhedral cells, iridocytes, which in the dog contain zinc cysteine crystals with reflective properties. The reflectivesness is responsible for the "eyeshine" seen at nigth, when a lightsource is directed at the animals face. The tapetum is roughly triangular in shape and covers approximately 30% of the superior fundus (see figure 2.1). It usually consists of 18-20 layers of cells, being thikest at the center of the tapetum and thinning towards the periphery and around the optic disc (Samuelson, 2007).

Puppies are born with an immature tapetum. It develops over the first 3-4 months of life and during this time it changes colour. It starts out being grey, then violet, then blue and finally

the adult coloration (see figure 2.2). The adult coloration show great inter- and intra breed variation, rainging from yellow-green to orange. In the Sable Collie and the Shetland Sheepdog the tapetal fundus is orange (Barrie, et al., 1981; Ollivier, et al., 2004; Samuelson, 2007).

The function of the tapetum lucidum is essentially like a mirror. Incoming light, that initially haven't been absorbed to by the photoreceptors reach the tapetum lucidum. Here it is reflected thus restimulating the photoreceptorers. This allows the animal to utilice as much light as possible present in any given situation, thereby improving the animals scotopic vision (Samuelson, 2007; Maggs, 2008; Miller, 2008).

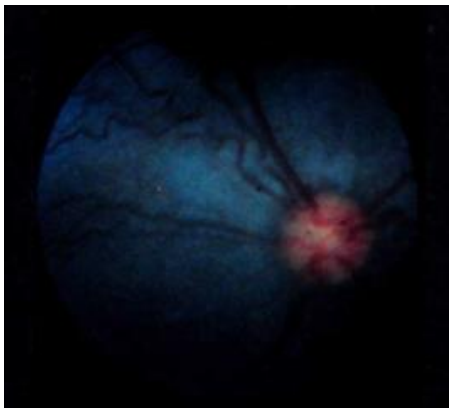

Figure 2.1 Immature fundus of a puppy. (Bedford, 1982a)

Figure 2.2 Mature canine fundus with tapetum lucidum. (Schoster, 2006)

3.3 Retina

The retina is commonly divided into the ten layers listed below (Samuelson, 2007).

- Retinal pigmented epithelium (RPE)
- Photoreceptor layer (contain the outer segments of the rods and cones)
- External limiting membrane
- Outer nuclear layer (contain the cell bodies of the rods and cones)
- Outer plexifom layer (synapses)
- Inner nuclear layer (contain the cell bodies of the secondary neurons)
- Inner plexiform layer (synapses)
- Ganglion layer (contain multipolar and smaller autonomic neurons)
- Optic nerve fiber layer
- Internal limiting membrane

The RPE lies just beside the choroid and is different in composition in respectively the tapetal- and the non-tapetal areas of the fundus.

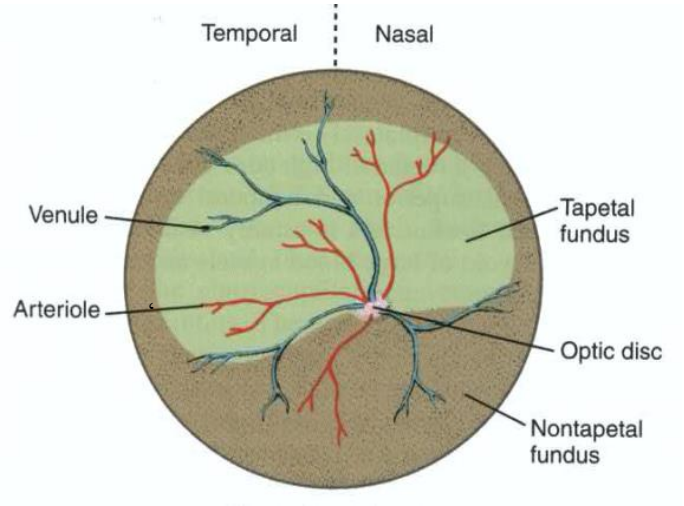

Figure 3.1 Schematic presentation of the canine fundus. (Maggs, 2008)

The RPE overlying the non-tapetal area is pigmented giving it a uniform brown-black color, which is why it is also sometimes referred to as the tapetum nigrum (see figure 3.1)(Samuelson, 2007). Like the tapetum of the choroid, the RPE is immature when puppies are born. It undergoes a maturation process during the first 12 weeks of life which can be seen as a change in it coloration. The color change represents the process of pigment embedding in the RPE (Hardon, 2015).

The RPE in the tapetal area is normally unpigmented and cannot be seen upon clinical examination (see figure 3.1). The lack of pigment allows light to reach the tapetum lucidum which was described in detail in the section above.

The main function of the RPE is to support the retina. It serves as a metabolic intermediary between the photoreceptors and the choriodal blood supply, by delivering nutrients to retina and removing waste from the outer part. The RPE also recycles “used” photopigment which, together with the ongoing production of new photopigment by the photoreceptors, play a vital role in the retina´s response to light (Orfri, 2008).

The other nine layer of the retina is collectively called the neuroretina. As the name implies, it is within these layers that the conversion of photic energy into neuronal signals takes place. For the purpose of this study, only the overall processes and most relevant structures are described.

The photoreceptor layer holds the outer segments of the photoreceptors, which contain the photo pigment. This is where the conversion of a visual input into a neuronal signal is initiated. The process is called phototransduction (Samuelson, 2007).

After the phototransduction process has begun, the neuronal signal is transmitted in a stepwise manner first from the outer nuclear layer to the neurons of inner nuclear layer. The outer nuclear layer contains the cell bodies of photoreceptors. There are two types of photoreceptors, the rods and cones, and each type has a specific set of functions. Rods are highly sensible to small changes in illumination, thus making them the visual cells primarily used at night (i.e scotopic vision) and in other situations of low levels of light. Furthermore they are also responsible for the processing of motion. Cones, on the other hand, are less sensitive and function best at high levels of light such as during the day (i.e photopic vision). They are capable of better visual discrimination thereby providing better acuity (Samuelson, 2007; Maggs, 2008; Orfri, 2008).

After the signal has travelled through the outer and inner nuclear layers, it continues further to the ganglion layer and finally via the nerve fiber layer to the optic nerve and brain (Wilcock, 2007)

3.4 Optic disc and Optic nerve

The extensive net of axons in the nerve fiber layer gather at a special area called the optic disc or optic papilla. In dogs, it is often pink in colour due to the presence of myelin, and round to triangular in shape. It is common to see variation in the size, shape and color of the optic disc. Differences in appearance are caused by individuality in the extent of myelination. Upon fundoscopy, the disc can be seen either in the tapetal area, the non-tapetal area or in their junction depending on the distribution of the two areas (Orfri, 2008).

Within the disc, a darker spot may be seen. This spot is called the physiologic cup and represents the origin of the embryonic hyaloid vasculature (Maggs, 2008; Orfri, 2008). This is considered a normal finding, and is not to be confused with a small coloboma.

From the optic disc, the nerve fibres continue in the optic nerve. The optic nerve enters the skull through the optic forarmen. In dogs, 75% of fibres cross to the contralateral side at the optic chiasm. Fibres from the temporal retina generally remain on the ipsilateral side of the brain, while fibres from the nasal retina cross over. If an eye is rendered blind, binocular

vision is lost, but stimuli from the visual field is continuously processed by the remaining eye. In these cases, fiber overcrossings therefore allow for both hemispheres of the brain to continue to receive visual input (Orfri, 2007).

Upon leaving the optic chiasm the fibres continue in the optic tract and terminate in the visual area of the cerebral cortex (Liebich and König, 2007). Here the final processing of the visual stimuli take place and become vision.

4 CEA

Collie Eye Anomaly is an inherited, congenital disorder which manifests itself as defects in the posterior segment of the eye. The defects are non-progressive and always bilateral, but commonly the two eyes are not equally affected. How the anomaly is expressed in very variable among individuals. The lesions range from mild, with only a small area affected, to large defects involving a significant part of the fundus and the optic disc. Visional effects range from no obvious impairment to complete blindness (Barnett, 1979; Bedford, 1982a).

Since CEA was first described, different criteria have been used to make the clinical diagnosis. Some of the earlier studies included chorioretinal dysplasia (CRD), coloboma/scleral ectasia, retinal detachment, intra ocular hemorrhage, tortuous retinal vessels, vermiform streaks and congenital corneal opacities as being part of the CEA-syndrome (Roberts, et al., 1966b; Roberts, 1969). Also enophthalmia and microphthalmia have been associated with CEA (Barrie, et al., 1981).

Over time, experts have discussed these criteria and attributed them different values to the CEA-complex. CRD and coloboma are the only two lesions which consistently seem to be accepted as indicative of CEA (Barnett, 1979; Barrie, et al., 1981; Wallin-Håkanson, et al., 2000a; Maggs, 2008; Miller, 2008).

4.1 Clinical pathology of CEA

4.1.1 Chorioretinal dysplasia(CRD) / Choriodal hypoplasia (CH)

The most common defect seen in CEA is focal “pale areas” seen in the fundus, typically situated temporal to superotemporal to the optic disc, and usually at the transition between the

tapetal and non-tapetal areas. They are areas with a lack of pigment in the RPE, the choroid or both (Barnett, 1979; Barrie, et al., 1981. The extent of the "pale area" is variable, ranging from mild (figure 4.1) to severe (figure 4.2) depending on the size of the affected area and the involvement of the optic disc.

In some severe cases, lesions can also be seen on the medial side of the optic disc, but always accompanied by a superior lesion on the temporal side (Barnett, 1979; Barrie, et al., 1981).

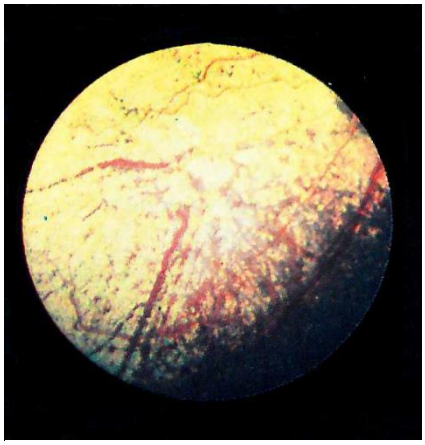

Figure 4.1 Minor CRD. (Bedford, 1982a)

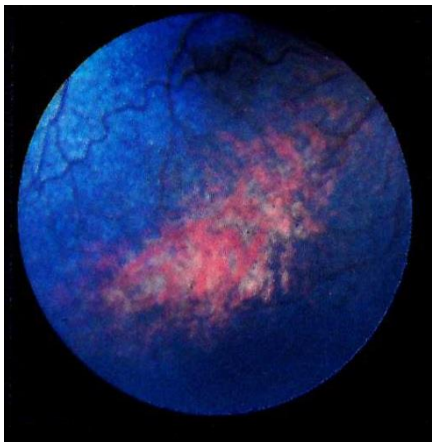

Figure 4.2 Severe CRD. (Bedford, 1982b)

In viewing of literature on CEA, authors have used different terms to describe the "pale area". *Chorioretinal dysplasia* (Barnett, 1979), *chorioretinal hypoplasia* (Martin, 2005) and *choriodal hypoplasia* (Bedford, 1982a) have all been used interchangeably to describe this same ophthalmological lesion. Some authors (Blogg, 1970; Bedford, 1982a) suggested, that choriodal hypoplasia (CH) is the more correct term to use. The argument for this suggestion was that the only retinal abnormality seen in animals with minor defects is a lack of pigment in the RPE. The retinal cells are otherwise normal in structure and function and therefore not dysplastic. Probably due to this argumentation, "choroidal hypoplasia" has later been accepted by both The European- and the American College of Veterinary Ophthalmologists (Genetics Committee of the American College of Veterinary Ophthalmologists, 2013; ECVO HED Committee, 2013).

Upon histological examination of CRD affected eyes, the choroid is generally found to be thinned and containing a reduced number of melanocytes, containing a limited amount of pigment. The tapetum lucidum is absent in the "pale areas" and reduced in thickness (2-3 layers of cells) elsewhere in the fundus (Roberts, et al., 1966b).

Abnormal choroidal vasculature in the “pale areas” is one of the primary lesions used to make the CEA diagnosis. Fundoscopic examination reveal vessels which are less numerous and broader in structure compared to normal (Barnett, 1979). Studies have shown poor blood-filling of the abnormal choroidal vessels compared to those in the rest of the unaffected uvea (Roberts, 1969)

Retinal abnormalities are, like those seen in the choroid, different depending on the degree of affliction. As described previously, in the mildest cases of CRD the retinal cells appear normal in function and structure. In more severe cases, vacuolation of the ganglion cells and thinning of the optic nervefiber layer have been demonstrated. In a report on the correlation between clinical –and histological findings in dogs with CEA, (Roberts, et al., 1966b) found that some dogs (aged 2 years or more) had degenerative changes within the retina which were not found to be typically associated with the normal ocular aging process. Histology revealed thinning, edema and cyst formation within the inner layers of the retina and also a reduced number of ganglion cells. In the RPE of some dogs, they found hypertrophied and hyperplastic cells with resulting displacement of adjacent patches of degenerated rods and cones. Attenuation of the outer nuclear layer and absence of rods and cones near the posterior pole was also found in the eyes of multiple individuals (Roberts, et al., 1966b).

4.1.2 Coloboma

The “hole-like” defects sometimes seen in dogs with CEA are most commonly referred to as colobomas. Other terminologies include “staplylomas” and “posterior scleral ectasia”.

As in the case of CRD these lesions vary a lot between individuals. They range from small/or shallow pits seen as grey indentations only occupying part of the optic disc, to larger and deeper areas involving all of the optic disc (see figure 4.3). Occasionally, extensive colobomas involve a considerable part of the posterior fundus much larger than the optic disc. Such severe lesions have a cystic appearance with the optic nerve pushed to the side (Barnett, 1979).

In clinical descriptions of the colobomas, they are categorized according to their position relative to the optic disc. Position on the optic disc is referred to as being papillary and adjacent to the optic disc as peri-or juxtapapillary. Futhermore, they are divided in typical

(located ventronasal/six o'clock position to the optic disc) or atypical (elsewhere in the fundus) colobomas (Bedford, 1982a; Martin, 2005).

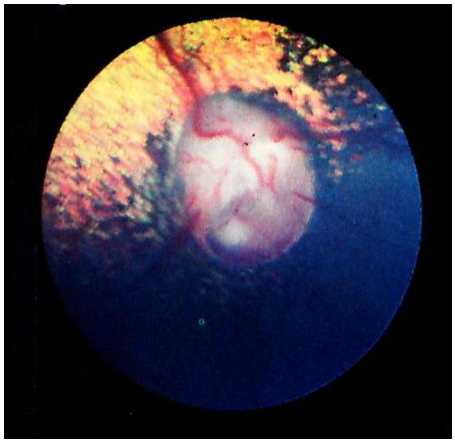

Figure 4.3 Typical optic disc coloboma. (Bedford, 1982a)

When examining a coloboma, it is sometimes possible to see blood vessels disappearing over the edge representing their descent into the pit (Barnett, 1979; Maggs, 2008). This feature may be useful when differentiating between a small colobomas and the physiological cup mentioned earlier.

4.1.3 Tortuous retinal vessels

Over the years, debate has taken place as to the significance of retinal vessel tortuosity to the CEA-complex. Some authors (Roberts, et al., 1966b; Yakely, 1972) have accepted, while others have questioned (Blogg, 1970) or rejected (Bedford, 1982a) the involvement.

Yakely (1972) suggested, that excessive tortuosity of the primary retinal vessels is to be considered indicative of CEA. This conclusion was based on results of breeding trials. Collies with excessive tortuosity of the primary retinal vessels as the only ophthalmologic finding were mated with Collies with CEA (CH, colobomas or both) and all progeny produced where affected by CEA.

On the other hand, Bedford (1982a) found tortuosity of the superficial retinal blood vessels in both affected and non-affected dogs, thus indicating that this clinical symptom was not diagnostic of CEA. Remarkably, it was noted that tortuosity of the primary retinal vessel only tended to accompany choridal hypoplasia.

Based on the before mentioned, whether or not excessive tortuosity of the retinal vessel is part of CEA or not isn´t clear. Collies in general have a higher degree of retinal vessel tortuosity compared to other breeds and the assessment of what is normal and what is excessive may

therefore be complicated (Barnett, 1979). Newer studies however, do not consider excessive tortuosity pathognomonic of CEA (Bjerkås, 1991; Wallin-Håkanson, et al., 2000a).

4.1.4 Secondary Changes

Intra ocular hemorrhage and retinal detachment are viewed as secondary changes in the CEA complex. Retinal detachment have been reported to occur in 6% of affected dogs and is therefore a relatively rare complication to CEA (Bedford, 1982a). Nonetheless, it is of great significance because of its devastating effect on vision. Lesions can sometimes be observed from an early age (6-7 weeks) in which case they are presumed to be congenital. Other times, detachment develops later but most often within the first year of life (Barnett, 1979).

Retinal detachments have generally been reported to occur in more severely affected eyes and often accompanying colobomatous defects (Roberts, et al., 1966a). Often detachments are unilateral and in most cases separation is total (Bedford, 1982a). A number of theories on the pathogenesis of retinal detachment in CEA affected eyes have been suggested. One theory describes the primary cause to be accumulation of subretinal fluid. The concurrent lack of support from the adjacent liquid filled vitreous makes an outwards protrusion of the retina possible (Barrie, et al., 1981). Another theory suggests colobomatous defects as a frequent place of origin, due to the lack of mechanical support and poor retinal nourishment which exist in these areas (Roberts, et al., 1966a).

Intraocular hemorrhage is likewise rare in affected animals. A prevalence of only 1% of CEA affected dogs have been found (Bedford, 1982a). The pathogenesis has been reported to be either due to rupturing of retinal vessels at the time of retinal detachment or as a result of bleeding from preretinal vascular loops (Roberts, 1969; Bedford, 1982a).

4.2 Vision in CEA affected dogs

As mentioned in previous sections, the visional effects of CEA range from no apparent clinical visual deficits to complete blindness and are dependent on severity of affliction. (Blogg, 1970; Barnett, 1979; Walser-Reinhardt, et al., 2009). Generally, severe visual impairment has been associated with retinal detachments, intra ocular hemorrhages and more extensive optic nerve colobomas (Donovan and Wyman, 1965).

Most CEA-affected dogs however have milder degrees of the anomaly and therefore no clinically observable loss of vision (Donovan and Wyman, 1965). Probably on this background, a detailed evaluation of vision in CEA affected dogs is not standard and therefore

not included in most medical records. The result is a very limited amount of data on the subject.

During the review of CEA associated literature, only one study of the visional effects of CEA was found. This study by (Donovan and Wyman, 1965) intended to evaluate the visional function by obstacle course testing of dogs with varying degrees of affliction. Like others, they found that loss of vision was primarily associated with retinal detachment and intraocular hemorrhage. Interestingly, they further stated that dogs with lesser degrees of affliction, such as CRD, would undoubtedly have some sort of visional impairment although clinically in evident (Donovan and Wyman, 1965).

In another paper on CEA, it was briefly mentioned how placement of an eye-patch over the less severely CEA-affected eye, may help reveal the actual degree of visual impairment of the contralateral, more affected eye (Blogg, 1970). Such testing led the author to report that vision in affected eyes may be much less than the owner suspects.

4.3 Embryologic development of CEA

Embryologic development of the eyes begins with two lateral outgrowths from the forebrain, named the optic vesicles. These vesicles invaginate thereby forming the optic cups, the choroid fissures and the optic stalk. The invagination gives rise to two adjacent layers of cells which will later develop into the pigmented outer layer (RPE) and the inner sensory layer of the retina (McGeady, 2006). The developing RPE governs the process of melanogenesis which is important relative to proper development of both the retina and the choroid (Strauss, 2005; Wilcock, 2007).

The two basic abnormalities seen in CEA (choriodal hypoplasia and coloboma) have been suggested to emerge from the same fundamental defect, a failure of inductive chemical signaling from the developing RPE. The inadequate stimuli provided by the RPE causes incomplete development the mesenchymal tissue designated to become the choroid, which leads to the hypopigmentation and hypoplasia seen in affected eyes (Wilcock, 2007).

Colobomas develop as a result of failed closure of the optic fissure, brought on by arrested growth of the fibrous tunic of the sclera. The growing retina is hereby allowed to bulge

outwards through the unclosed fissure thus permanently preventing the closure of the embryonic fissure and sclera (Wilcock, 2007).

Histological studies of CEA affected embryos have also found a possible association between prenatal rosette formation and later development of colobomas (Roberts, et al., 1966a; Latshaw, et al., 1969). The source of rosette formation was suspected to be dilation or faulty obliteration of the optic vesicle. This could lead to disturbances of the interaction between the inner and outer layers of the optic cup which is supposedly necessary for proper differentiation (Latshaw, et al., 1969).

4.4 Clinical diagnosis

Clinical diagnosing of CEA is done on the basis of an ophthalmoscopic examination of the fundus. The examination is performed in a dark examination room and starts with an examination without pupil dilation. Thereafter, the pupils are dilated by the use of a mydriatic such as tropicamide. Often, the fundus is examined by using both indirecte- and directe ophthalmoscopy (see figures 4.4 and 4.5). Most ophthalmologists prefer to start with an evaluation of the fundus as a whole by exploiting the greater field of view offered by the indirect ophthalmoscope. Thereafter they use the higher magnification power of the direct ophthalmoscope to examine potential anomalies more closely (Maggs, 2008).

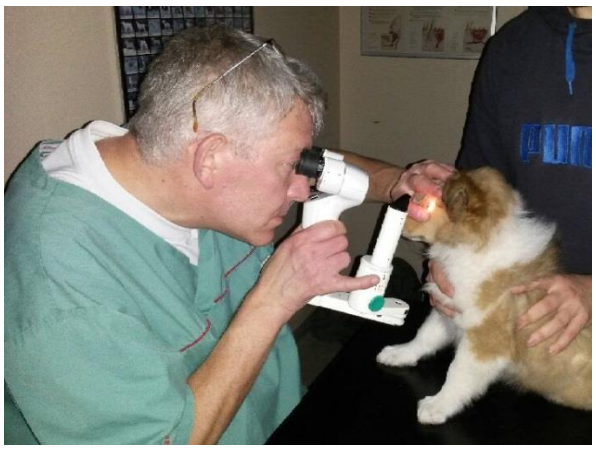

Figure 4.4 Direct opthalmoscopy

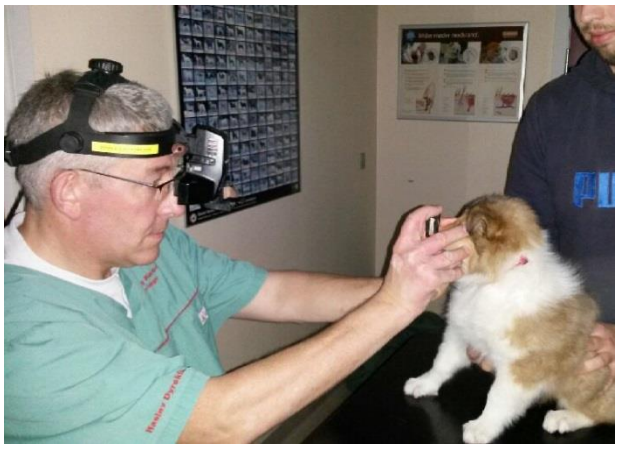

Figure 4.5 Indirect opthalmoscopy

In the clinical diagnosing of CEA it is of great importance, that the ophthalmologic examination is performed early, preferably between 5-10 weeks of life. The reasoning behind

this recommendation is to avoid the well documented "go-normal" effect (Bedford, 1982a; Bjerkås, 1991). "Go-normal" is an expression used to cover the phenomenon where minor choroidal hypoplastic changes diagnosed in young puppies seem to have disappeared when they are examined later in life. The lesions, however, have not disappeared but have only been masked by the previously described process of delayed pigment embedding in RPE. The significance of the "go-normal" effect has been reported by Bjerkås (1991). He found an occurrence of 68 % (15/22) in dogs diagnosed with minor CRD changes.

Examination of such young individuals can however sometimes present a challenge. The relatively small size of the eyes combined with the lively tempers of puppies can make adequate restrain almost impossible (Bedford, 1980).

The presence of merling is also a factor which potentially complicates diagnosis (Yakely, 1972). Many merle dogs have complete or partial subalbinotic fundi which represents the naturally occurring lack of pigment in these individuals. In completely blue eyes ("china eyes"), all of the choriodal vasculature is visible with the white sclera as a background. Also the overlying broader and paler red retinal vessels can be seen (see figure 4.). In partial blue (heterochromic eyes) the pigmentation is nearly normal in most of the fundus except from pigment depleted patches within the RPE, the tapetum lucidum or both. These patches allows for the underlying choroidal vasculature to be seen (Barnett, 1979).

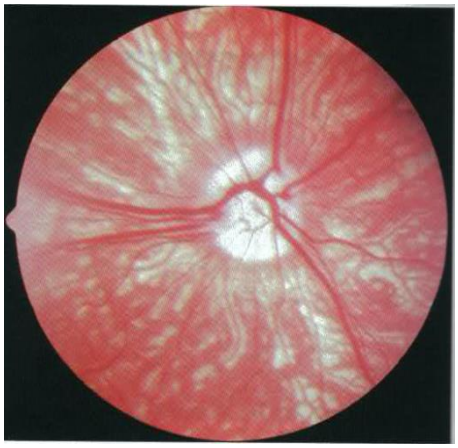

Figure 4.6 Subalbinotic fundus. (Maggs, 2008)

5 Genetics

Gregor Mendel was the founder of the Mendelian laws of inheritance which formed the basis of modern science of genetics. Among other things, he found that certain traits were only expressed if the individual inherited two copies of the same allele i.e. the alleles were

recessive. He also showed how test- and back crossings could reveal the mode of inheritance of biological trait (Hartwell, et al., 2008).

Before the times of genetic testing and discovery of the canine genome, the study of presumably inherited diseases relied on these principles and methods.

5.1 Genetics of collie eye anomaly

From time where the ocular lesions, later known as Collie Eye Anomaly, was first described by Magrane in 1953, many ideas regarding the mode of inheritance of CEA have been proposed.

During the 1960's CEA was intensively studied with the intent to shed light on both the clinical aspect of the anomaly and the mode of inheritance.

One of these early studies, done by Donovan and Wyman (1965), intended to gain further insight into the genetics of the anomaly. They suspected the CEA-complex to be of an inherited nature supported by fact that inbreeding seemed to intensify lesions whereas outbreeding seemed to dilute them. On the basis of pedigree analysis and breeding trials they further suggested a possible polyfactorial autosomal recessive mode of inheritance. Further support of an autosomal recessive mode of inheritance was found in successive studies (Yakely, et al., 1968; Donovan, et al., 1969).

Yakely, et al. (1968) also found that the anomaly was not sex-linked due to similar frequency in both males and female. They reflected on the variability of expression of the anomaly and proposed the theory of genes at more than one locus having effect on the phenotype.

Another theory was argued by Roberts and Dellaporta (1965). They proposed the idea of one single defective factor with variable penetrance and possibly also pre-and post natal degenerative changes, to be the source of the very variable clinical presentation of the anomaly. They hypothesized the hereditary factor to be one which affected pigmentation and ocular growth (Roberts, et al., 1966b) and further studies let them to conclude the factor was the gene for merling (Roberts, 1967).

This conclusion was however questioned later by Rubin (1969). He investigated the prevalence of CEA in Collies of different coat colors and could not demonstrate any significant difference between merled and non-merled populations (Rubin, 1969).

As could be drawn from the above, a certain degree of controversy on the genetics of CEA existed at the end of the decade. The collected data on CEA was therefore discussed by the panelists of the American Society of Veterinary Opthalmology at a symposium in September 1969, with the purpose of arriving at some sort of consensus about what the recommendations to owners and breeders of Collies should be. Primarily, thoughts on the mode of inheritance of the various lesions associated with CEA and the long term effect of breeding only slightly affected animals were argued (Catcott, 1969).

At the symposium, all agreed upon a simple autosomal recessive inheritance of the primary CEA phenotype, CRD (Catcott, 1969). An independent mode inheritance of staphyloma (coloboma) was also a subject of discussion. This theory was suggested by Donovan, et al. (1969) on the basis of results obtained from breeding trials. The results showed a ratio of affected to non-affected which excluded a simple autosomal recessive mode of inheritance for coloboma. Instead, it pointed at a simple autosomal dominant transmission (Donovan, et al., 1969). This mode of inheritance was however not accepted by all of the participating panelists (Catcott, 1969).

In the years following, work on CEA did not question the autosomal recessive mode of inheritance (Blogg, 1970; Yakely, 1972; Bedford, 1982a; Bedford, 1982b). Non-genetic factors such as intrauterine environment affecting the phenotypic expression were discussed, but no conclusions were made (Yakely, et al., 1968; Yakely, 1972).

In the year 2000, an extensive study by Wallin-Håkanson, et al., (2000a) investigating the prevalence of CRD and coloboma in Swedish rough collies over a period of 8 years, was published. In this period, the Swedish breeders had avoided breeding dogs with coloboma and only bred mildly CRD affected dogs. The results of the study showed a relative increase in the prevalence of CRD while the prevalence of coloboma decreased. This result led the authors to propose separate modes of inheritance for CRD and coloboma which was in accordance with the results found by (Donovan, et al., 1969).

Wallin-Håkanson, et al (2000b) also investigated litters in which both parental and offspring CEA status were known and compared the observed phenotype frequencies with those expected under a simple autosomal recessive mode of inheritance. There was a significant

difference between the observed and expected frequencies on which basis a polygenetic mode of inheritance was suggested. Interestingly they also found a positive correlation between reduced offspring vitality and the presence of coloboma in one or both of the parental animals.

The conclusions made by Wallin-Håkanson, et al.'s (2000a,b) was commented on by lecturer David R. Sargan in 2001. Dr. Sargan argued that the results found could in fact be explained under a simple autosomal recessive mode of inheritance if the defect either had incomplete penetrance, modifier gene loci or was influenced by environmental factors or "breed-wide effects in the genetic background". Based on his argumentation, he had hope for development of a genetic test for CEA by finding a "single gene of major effect". B. and N. Wallin-Håkanson responded by arguing that Dr. Sargan bringing up incomplete penetrance etc. in fact supports their theory of a polygenetic mode of inheritance in which several gene loci would be involved (Sargan, et al., 2001).

5.2 Genetic mapping of CRD

Approximately 15,000 years ago, man began domestication of wolves. Subsequently, highly selective breeding have led to the development of more than 400 individual dog breeds possessing very different morphological and behavioral characteristics e.g. herding, guarding and hunting (Wells, 2002).

Purebred dogs are subjected to intense selection based on a specific set of breed standards which combined with the fact that many breeds can be traced back to a very limited number of forefathers and foremothers, makes purebred dogs members of essentially closed breeding communities (Sutter and Ostrander, 2004).

Over the last decades, the potential uses of the before mentioned unique population structure within breeds have been recognized. Comparative maps incorporating both the canine- and the human genome have been constructed, and such maps are used to identify candidate genes and genetic mutations causing disease in both humans and dogs (Lowe, et al., 2003).

The first attempt to genetically map CRD was done by Lowe, et al. in 2003. They used the before mentioned comparative human-canine genome maps to investigate segregation of CRD. By recording LOD scores between CRD and different markers, the candidate area was found to be a 3,9 cm region of canine chromosome 37. Despite their conclusion of allelic

mutations at a single locus being responsible for the expression of CRD, they discussed the possibility of independently segregating modifier loci being the cause of the "go-normal" effect and thereby interfering with clinical identification of CRD. They acknowledged the wide range of phenotypic expressions seen in CEA affected dogs and suggested that the entire CEA complex is caused by more than one genetic contributor. Interestingly, they also reported a CRD penetrance of less than 100% in homozygous affected dogs and partial penetrance in heterozygous affected dogs.

Lowe, et al. did not identify the specific gene responsible for CRD and therefore further studies were needed in order to narrow in on the causative gene (Lowe, et al., 2003). Such studies were later performed by Parker, et al. in 2007.

Parker, et al (2007) started their article by explaining how it can be challenging to identify specific genetic sequences in the dog due to the fact that members of a single breed have large, continuous segments of DNA in common. They further explained how identification of allelic mutations in multiple breeds can reduce the size of the region of interest by taking advantage of shortened linkage disequilibrium distances found across breeds.

In order to use the multiple breed approach, they used cluster analysis to group 132 distinct breeds into five main breed groups. Breeds within the same breedgroup were expected to have common ancestry and therefore share certain traits. The four breeds, Collie, Shetland Sheepdog, Australian Shepard and Border Collie in which the prevalence of CEA has been well established, were all found to belong to the herding/sighthound cluster. The clustering together of these breeds led the scientists to speculate a shared causative mutation identical by descent.

Based on the previous localization of the primary CEA defect (CRD) to canine chromosome 37, the Parker, et al. group first designed markers to fine map CRD under the assumption of a similar gene order and content existed between the CRD interval of this chromosome and the homologous region on human chromosome 2. Later, in 2005, the release of the 7,5 × Boxer sequence assembly (CanFam1) and a SNP database permitted for more markers to be tested. The result was a reduction of the shared haplotype to a 102,495 bp region.

Hereafter, a BAC contig map across the CRD interval was constructed. PCR analysis, with SNPs and other markers, of the BAC contig led to the discovery of the entire CRD linkage

disequilibrium (LD) being included in a single BAC clone. The LD interval was defined by a SNP haplotype shared by all tested CRD affected chromosomes. The shared haplotype contained four genes, one of which was the *NHEJ1* gene. Sequence analysis of flanking- and coding regions (exons) was performed but did not expose a potential causative mutation. A potentially significant intronic mutation was detected in all affected chromosomes. The presented mutation was a 7799-bp deletion within intron 4 of the *NHEJ1* gene.

After having found the deletion, assessment of its occurrence in a broader population of dogs was needed. Samples from 90 dogs (58 cea-affected and 32 obligate heterozygotes) belonging to four breeds known to segregate CRD were tested for the deletion by using a two-step PCR. The primers used were placed across and within the deletion in order for quick identification of chromosomes with and without the deletion. Furthermore, 93 dogs from 45 breeds not known to segregate CRD were tested. Results showed that all 58 cea-affected dogs were homozygotes and the 32 obligate heterozygotes all carried the mutation at only one of the chromosomes.

The deletion was found in only one chromosome of the 93 dogs from presumably unaffected breeds, a Boykin Spaniel. 16 additional Boykin Spaniels were tested and another five dogs were found to be heterozygous for the mutation. Subsequently, a smaller number of Boykin Spaniels segregating the CRD phenotype was identified as homozygous for the deletion thus establishing the connection between the anomaly and the mutation in this breed.

Additional testing for the deletion was done in several other breeds in which CRD, or a CRD-like phenotype had been demonstrated. Lancashire Heelers, Nova Scotia Duck Tolling Retrievers and Longhaired Whippets diagnosed with CRD were all found to be homozygous for the deletion; obligate carriers which were clinically unaffected by CRD, were heterozygous for the mutation. Dogs from the Berger des Pyrenees breed which were clinically diagnosed with coloboma were likewise tested for the mutation, but these individuals did not have the deletion. A group of Soft Coated Wheaten Terriers expressing a phenotype similar to CRD was also tested, but the deletion was not detected.

In their discussion, Parker, et al. explain that the deletion contains highly conserved segments with binding sites for a number of regulatory proteins. They suggest that interaction between

such proteins and the conserved regions affects embryologic development of the eye and is thereby causative of CRD.

Parker, et al. go on to discuss that the study results indicate that the mutation arose as a single disease allele and were thereafter spread to many herding breeds when these were developing. However, the mutation was also demonstrated in other breeds (Lancashire Heeler, Longhaired Whippet and Nova Scotia Duck Tolling Retriever) which did not cluster with the herding breeds. An explanation of the mutation in the Lancashire Heeler and the Nova Scotia Duck Tolling Retriever was found in the individual breed histories, which seemingly offer evidence of collies being bred into the genetic lines at an early time. The Longhaired Whippets were also supposed to have long-coated herding dogs in their lineage but no documentation was presented.

5.3 Genotyping by the use of polymerase chain reaction

The current DNA test for CRD seeks to find whether or not the tested individual carries a 7,8 bp deletion in the NHEJ1 gene. By using PCR one can rapidly amplify large amounts of the DNA-region of interest and thereafter determine the genotype.

Before commencing the actual PCR reaction, one must first prepare a sample of DNA and add to this a mix of components needed to perform the amplification. Some of the very important components are the primers. Primers are specific, singlestranded oligonucleotides which bind at sites flanking the DNA sequence of interest and thereafter serve as the starting point for synthesis. One of the primers base pair in one end of one of the single-stranded DNA-templates, while the other primer base pair at the opposing end of the other single-stranded DNA-template. Primer sets have a forward and a reverse primer indicating the direction of which synthesis takes pace. In addition to the primers, a mix of the four nucleotides (guanine, cytosine, thymine and adenine) and a specialized DNA-polymerase are needed. To ensure the optimal environment for the reaction, a buffer solution is also added (Hartwell, et al., 2008).

The PCR-test tubes containing the described components can then be placed in the thermal-cycler and the amplification process can begin.

The process can be divided into five steps: 1) initialization, 2) denaturation, 3) annealing, 4) elongation and 5) final elongation (Hartwell, et al., 2008).

The following description of the process is based on the PCR-program used in our study.

Step 1) Initialization:

In this first step, the mixture is heated to activate the DNA-polymerase. The temperature and time needed depends on which polymerase is used. In our study we used *Taq* polymerase and therefore the mixture was heated to 94°C for 15 minutes.

Step 2) Denaturation:

The temperature was kept at 94°C for 30 seconds. This separates the two DNA-strands by breaking the hydrogen bonds between the complementary bases. The result is two single-stranded DNA strands which will serve as templates.

Step 3) Annealing:

The temperature was lowered to 60 °C for 30 seconds. This allows for the primers to base pair with the individual single-stranded DNA-templates as described above. After biding of the primers, *Taq* polymerase binds to the primer-template hybrids and DNA construction can begin.

Step 4) Elongation:

The temperature of this step also depends of the polymerase selected. We used *Taq* polymerase and therefore was the temperature raised to 72°C. The polymerase starts synthesizing the new DNA strand complementary to the template strand, by adding nucleotides from the mix. The amount of time at this step depends of the length of the DNA fragment to be amplified. In our study it was 45 seconds.

Step 2-4 is repeated for a number of cycles. In ours it was 35 repeats.

Step 5) Final elongation:

This step is only performed after the last of the cycles. The temperature is held at the optimum for the polymerase for 15 minutes to ensure that all single-stranded DNA is fully elongated.

6 Breeding restrictions and recommendations

The mode of inheritance of CEA is supposedly the same in all breeds known to be affected (Parker, et al., 2007). In spite of this, breeding restrictions and recommendations differ quite substantially between affected breeds.

In Denmark, the DKK have a shared set of breeding restriction, in regards to eye examination, for both the Shetland Sheepdog and the Rough Collie. The breeding restrictions state: "*Puppies cannot be registered unless both parental animals, before mating, are examined for CEA by a member of the Danish Veterinary Association eye panel or another veterinary ophthalmologist approved by the DKK. The most optimal period for this examination is between 7.-9- weeks of age"* On the basis of this eye examination the dog will receive an ECVO approved eye certificate. The CEA status given to the animal applies for life (Dansk Kennel Klub[b]; Dansk Kennel Klub[c]).

In contrast, the Border Collie which is also known to be affected by CEA, do not have any DKK breeding restrictions relative to eye examination (Dansk Kennel Klub[a])

Interestingly, the breeding recommendations for the three breeds differ significantly concerning CEA. As of January 1st 2015 a new addition regarding CEA has been added to breeding recommendations for the Shetland Sheepdog. This addition was suggested by the Health Comiteé of the Danish Shetland Sheepdog Club. They state, that at least one of the parental animals, must by eye examination, be registered free of CEA before mating and if a DNA-status exist, it must also be free of CEA i.e. homozygous recessive (Dansk Kennel Klub[c])

The breeding recommendation for Border Collies state that both parental animals must have a DNA-test result for CEA prior to breeding. Carriers i.e. heterozygotes must only be mated with CEA free. Affected must not be used in breeding (Dansk Kennel Klub[a])

There haven't been issued any CEA breeding recommendations for Rough Collies, neither by the DKK nor by The Danish Collie Club (Dansk Collie Klub; Dansk Kennel Klub[b]).

Differences in CEA associated breeding recommendations are also found on more international levels such as within the two well recognized veterinary ophthalmology colleges, ACVO and ECVO.

The ECVO manual guidelines utter that breeding of CH/CRD affected dogs is optional for the breeder, whereas dogs with coloboma or retinal detachment/intraocular haemorrhage should be excluded from breeding (ECVO HED Committee, 2013). The ACVO Blue Book recommendation on the other hand, discourages breeding with all types of CEA affected dogs (Genetics Committee of the American College of Veterinary Ophthalmologists, 2013).

CASE CONTROL STUDY/QUESTIONNAIRE

7 Materials

7.1 Genotyping of Shetland Sheepdogs

In collaboration with DKK, a list of eye-examined Shetland sheepdogs registered between the years 2009-2014 was sent to The Health Committee of The Danish Shetland Sheepdog Club. This list contained the eye-health information of the dogs including their CEA status. The Health Committee was asked to select 50 dogs with a clinical CEA diagnosis and 50 dogs without. Furthermore the selected dogs should meet the following criteria:

1) The clinical eye-examination should be performed between 5-12 weeks of age
2) The dogs should be unrelated on parental level (i.e. no siblings)

The owners of the selected dogs received an envelope containing an official letter with a study description, a FTA card and a manual on how to collect a DNA-sample from the buccal mucosa (Appendix A), a sterile foam-tipped applicator and return-slip for sample identification. The FTA cards and matching identification slips were sent to the laboratory of The Department of Veterinary Clinical and Animal Sciences, Division of Genetics and Bioinformatics.

7.2 Questionnaire

A questionnaire containing 7 questions was sent to the owners of the 45 Rough Collies who participated in the previous CEA study. These dogs were selected on basis of the same criteria as those of this case-control study.

Also 30 Shetland Sheepdogs owners, those who wanted to receive the DNA-test results of their dogs, received the questionnaire.

8 Methods

8.1 Genotyping of Shetland Sheepdogs

The FTA cards were, upon arrival at the laboratory, assigned with an individual ID-number running from 1-56. Throughout the genotyping process, we did not know which dogs were in which samples and therefore the study was blinded.

Isolation of DNA from FTA-cards

The isolation of DNA from the collected FTA-cards was performed in accordance with the Whatman® protocol. For a detailed description see Appendix B, "Preparation of Sample DNA for Downstream Analysis".

PCR

In the section describing the PCR-reaction in general, it was explained that in order to amplify the alleles of a specific region of DNA, one must use a set of primers. In our study, we used the primer sets designed by Parker et al, 2007 to look specifically for the deletion within the *NHEJ1* gene (see figure 8.1).

The primer sets were as follows:

Primer I, F17/R17 within deletion:
NHEJ1-F17, 5´- TCTCACAGGCAGAAAGCTCA-3´
NHEJ1-F17, 5´- CCATTCATTCCTTTGCCAGT-3´

Primer II, F20/R23 across deletion:

NHEJ1-F20, 5´- TGGGCTGGTGAACATTTGTA-3´

NHEJ1-R23, 5´- CCTTTTTGTTTGCCCTCAGA-3´

Primer I amplifies the wildtype allele (CRD) if present. The reason why only the wildtype allele can be amplified, is because the binding site for the reverse primer (R17) is located within the deleted areas on chromosomes carrying the mutation. Binding of the primer is therefore not possible and no amplification will take place.

Primer II is placed across the deletion and will only amplify the mutant allele (crd). This is due to the fact, that the product for the wildtype is too large to be significantly amplified.

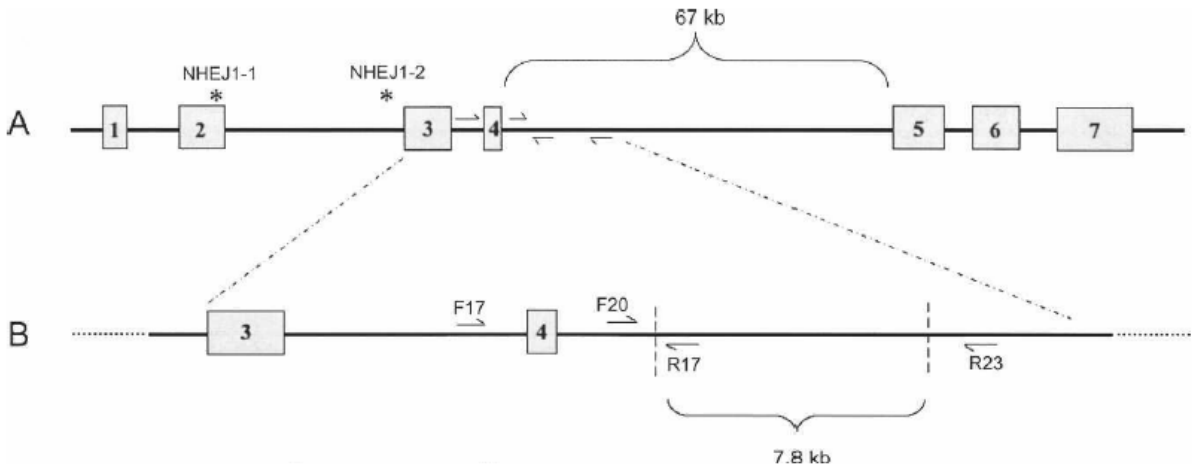

Figure 8.1 Illustration of the two-step PRC protocol used for genotyping of the CRD-associated deletion. (A) The *NHEJ1* gene and the linked SNPs designated by * and primer locations within intron 4. (B) Expanded representation of the CRD-associated deletion region, with flanking and internal primers. (Parker, et al.,2007)

PCR-program

The PCR-program used was as follows:

- 94 °C for 15 minutes (activates *Taq* polymerase)
- 94° C for 30 seconds (denaturation)
- 60° C for 30 seconds (annealing)

- 72° C for 45 seconds (extension)
- 72° C for 10 minutes (extension)
- 4-12° C (storage temperature)

The process was repeated for 35 cycles.

PCR Reaction

The mastermix used for the PCR reaction contained the following components:

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

We added 18 µl of mastermix, 2µl H_2O and 1 FTA punch/pellet to each PCR tube.

Gel electrophoresis

After PCR amplification, we separated the DNA-fragments by the use of gel electrophoresis. The gel used was 1,5 % agrose. One of the wells on the gel was loaded with water representing a "blank" sample.

Fragment separation is achieved when electrical current is passed through the gel during a period of ca. 30 minutes. The DNA fragments separate according to their size. Larger fragments travel slower on the gel thus separating after a shorter distance. Smaller fragments are quicker, thus travelling a further distance. The size of the fragments is assessed by the guidance of a DNA ladder (here we used a 100 bp ladder) (Hartwell, et al., 2008).

8.2 Questionnaire

The questionnaire was designed by the author in collaboration with Professor Merete Fredholm and Tommy Hardon (ECVO certified ophthalmology specialist). The electronic version was made via www.surveymonkey.com and distributed via an email-link. The paper version was sent to the owners for whom we did not have email addresses.

9 Results

9.1 Genotyping

From the 100 FTA-cards sent out, we received 55 containing DNA-material. 28 samples were obtained from clinically healthy dogs and 28 samples from dogs clinically diagnosed with CRD/CH. Out of the 28 dogs with CRD/CH, 2 also had coloboma, one had unilateral retinal detachment and one had intraocular hemorrhage.

After having run the gel-electrophoresis, we held the gel under a UV light source making it possible to read the amplified DNA-fragments. An example of one of the electrophoretograms is shown below. Electrophoretograms for all the dogs are found in Appendix C.

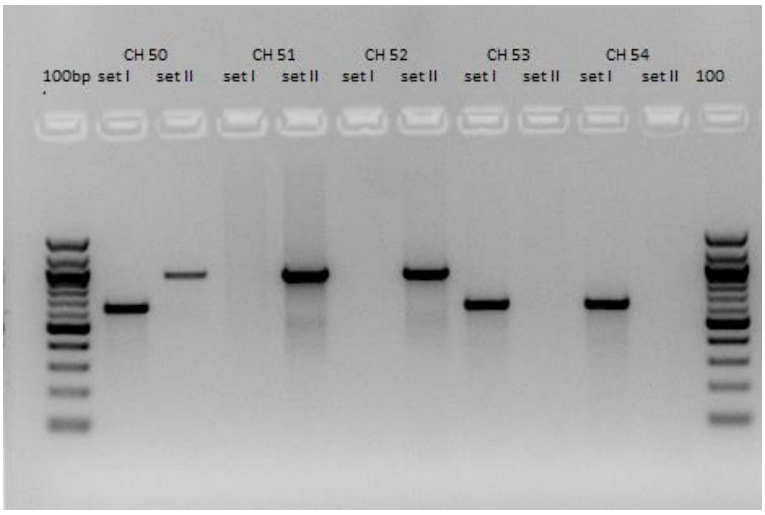

Figure 9.1 Electrophoretogram showing the PCR results of tested dogs CH 50-CH54. Set I contains primers F17 and R17. Set II contain primers F20 and R23. Testsubject showing only a band in the set I lane is homozygous for the wildtype (CRD/CRD). Testsubjects showing only a band in the set II lane is homozygous for the mutant allele (crd/crd) and those that have bands in both lanes are heterozygous (CRD/crd).

The individual results of genotyping can be found in Appendix D. The genotype- and allele frequencies were calculated and results are listed in table 9.1. The allele frequencies were calculated by the gene counting method as shown below.

Frequency of CRD allele = p = (2*16+15) / (2*56) = 0,42

Frequency of crd allele = q = (2*25+15) / (2*56) = 0,58

Table 9.1 Genotype- and allele frequencies in the study population. The wildtype allele is identified as (*CRD)* and the mutant allele is identified as (*crd).*

	Genotype Frequencies			**Allele Frequencies**	
	Homozygous *CRD/CRD*	Heterozygous *CRD/crd*	Homozygous *Crd/crd*	Wildtype *CRD*	Mutation *crd*
Shetland Sheepdog (n = 56)	16/56 = 0,29	15/56 = 0,27	25/56 = 0,44	0,42	0,58

9.2 Questionnaires

We received responses to the questionnaire from 21 of the Rough Collie owners and 14 of the Shetland Sheepdog owners. One of the collies was deceased and the questionnaire was therefore returned unanswered.

Upon reviewing the answers given by the owners, there was a high degree of uniformity between the two breeds. On this basis, the results will be interpreted as coming from a single source.

The individual replies to the questionnaires are found in Appendix E.

Question nr. 2

The owners were asked to state the precise age of the first eye-examination of their dog.

All dogs, but one, were examined in the period from 6 to 8 weeks age. A single dog was examined at 14 weeks of age.

Question nr. 3, 4 and 5

First, we asked the owners if their dog had any eye problems from the time of the first eye-examination till now. Then we wanted to know what the diagnosis was, if there was one, and what age the dog had at the time of diagnosis.

31, corresponding to 91%, of the owners replied that their dogs didn't have any eye problems in the period.

One owner answered, that the dog had conjunctivitis. Two owners misread the question and instead stated the diagnosis given at the first eye-examination.

Question nr. 6 and 7

We asked the owners if they experienced their dog to have normal vision, and if not what were the symptoms.

33 of the 34 owners (97%) responded that they perceived their dog to have normal vision. The one dog that stood out, was the same dog that had bilateral coloboma. The symptoms of inferior vision seen in this dog was wanting to walk the same way on walks, bumping in to things and getting scared if something new happened around it.

Question nr. 8

We wanted to know if the owners was using/ or planning to use their dogs for breeding.

13 answered “no” and 20 answered “yes”. One chose not to answer.

Discussion

In the study we found allele frequencies of respectively 0,58 for the mutant allele (*crd*) and 0,42 for the wildtype allele (CRD). It has not been possible for the author to find any studies similar to ours and therefore a discussion of results is complicated. The closest comparison is the study done by (Dostal, et al., 2010) which found a mutant allele frequency of 0,429 and a wildtype allele frequency of 0,57 in tested Shetland Sheepdogs. From these results one might be tempted to conclude that the Danish population of Shetland Sheepdogs generally has a higher frequency of the mutant allele compared to the Czech Republic.

Such an interpretation of the results would not be reasonable since there are some important differences in the study design which influence on the results. First, there is a significant difference in the composition of the two study populations. Our population consisted of individuals selected on the basis of their phenotype, while Dostal et al.'s was made up by randomly selected purebred dogs. This difference alone will most likely raise the allele frequency in our population compared to theirs.

Another important factor to consider is the sizes of the two populations. Dostal, et al. tested 141 Shetland Sheepdogs while we tested 56. Neither of the two sample sizes is large enough to produce results which, with statistical confidence, could be applied to the entire population. In other words, taking the results and applying them to a nationwide scale would be tied to high degree of uncertainty and therefore not advisable.

Our study on the compliance between the clinical diagnosis of CEA and the DNA-test for mutation in NHEJ1 gene suggest a high degree of compliance. This result agrees with that found by Parker, et al. (2007). Only in 5 out of the 56 dogs, corresponding to 8,9 %, were there disagreement between the two diagnoses.

These five dogs all shared the same coat color, blue merle, which might offer an explanation to the inconsistencies. Dogs with this particular coat color have been associated with difficulties in clinical diagnosing because they often possess a partial or complete subalbinotic fundus. One could therefore argue the possibility of clinical misdiagnosing in these individuals. In cases where the clinical diagnosis is uncertain, Parker, et al. (2007) suggests that the genetic test might help to avoid false diagnosis.

Interestingly we have conflicting results with those of the precursor thesis study done by Jönsson and Söderlund (2014) in Danish Rough Collies. In their study they found a very poor compliance between the clinical diagnosis and the DNA-test. They pointed out that the disagreement was found in the group of clinically healthy dogs, where all but one were genotypically affected i.e. homozygotes for the NHEJ1 gene mutation. Such a degree of divergence could not be due to chance. Jönsson and Söderlund discussed the possible causes to their poor compliance. The possibility of misdiagnosis, incomplete penetrance, modifier loci and the go-normal effect were all considered, but none of which seemed to offer them a likely cause to the almost total lack of compliance.

The obvious question raised in this discussion, is why is there such a difference between the two breeds studied?

The existing literature on CEA deals very little with both clinical- and genetical differences between affected breeds. The only description of a clinical difference between Rough Collies and Shetland Sheepdogs was found in the work on CEA in the UK, done by Bedford, (1982a). In his description of the clinical features in the Shetland Sheepdog, he notes that choroidal hypoplasia tends to be much more marked in this breed, compared to that which occurred in the Rough Collie. Rough Collies could therefore sometimes be more challenging to diagnose in cases where they only had minor lesions (Bedford, 1982a) . If this tendency is also true in the Danish Rough Collies, one might argue the possibility that some of the collies in the previous CEA study were misdiagnosed as clinically unaffected. Despite this suggestion, it seems unlikely that such a high number of dogs would be misdiagnosed.

Alternatively to a clinical source of the inconsistencies, it could be considered if in fact the mode of inheritance of CRD is not as clear developers of the genetic test expects. The recent work by Wallin-Håkanson, et al (2000b) strongly supports this theory.

When interpreting the validity of the compliances found, both in this study and the previous one, the effects of the relatively limited sample sizes must be weighed in. Neither of the studies offers statistical significant results and they must therefore be interpreted with caution.

The answers given in the questionnaire were rather uniform and some tendencies can therefore be drawn from them.

We showed that almost all of the dogs for which questionnaires were answered were eye-examined in the last week before leaving home at 8 weeks of age. If this result was representative of the entire population, it could be stated that the likelihood of dogs being misdiagnosed as "free" due to the "go-normal" effect would be very slim or non-exsisting.
Furthermore it would seem that all most all breeders are following the DKK breeding recommendations. To validate this tendency, the author reviewed all DKK registered Shetland Sheepdog litters produced in the year of 2013, to see if it was a general tendency. Unfortunately it was not so. From the 79 litters produced in the period, 20 litters (≈25%) were not eye-examined within the first 12 weeks of life (DKK^{d}). The underlying reasons why some breeders choose not to eye-examine within the recommended period, can only be speculated. Whatever the reason, it shows the importance for veterinarians and breeding associations to inform breeders of the implications of such a choice e.g. the possibility of "go-normal" dogs.

From the answers to question 3, 4 and 5 regarding eye problems which have emerged after the first eye-examination, it appears as if both Rough collies and Shetland sheepdogs do not have high frequencies of eye-problems aside from CEA. Only one dog had conjunctivitis which is considered a random finding. Rough Collies and Shetland Sheepdogs have other ocular disorders of both hereditary and non-hereditary nature e.g. districhiasis, ectopic cilia, PPM, PRA, PHTVL/PHPV etc. but a discussion of these lies beyond the scope of our study (Genetics Committee of the American College of Veterinary Ophthalmologists, 2013).

Within the limitations of this thesis, it was not possible to clinically investigate the vision in affected dogs. Instead we chose to approach the subject of vision from the angle of the owners.

From the answers to question 6 and 7, we can draw that both owners of dogs with and without a clinical CEA diagnosis experience their dogs to have normal vision i.e. there is no difference. Only one dog affected by coloboma, showed signs of visual impairment.
This result could be interpreted as vision being uncompromised in most CRD affected animals, which agrees with observations made in other studies (Blogg, 1970; Barnett, 1979; Bedford, 1982a).

The possibility of several factors influencing the result, should however be taken into consideration. First, one could argue that the perception of "normal vision" may be very diverse among the individual owners. Since we haven't listed any signs of inferior vision in the question, the answers are based entirely on subjective opinions. If in fact some affected dogs have visional impairment, such defects would be present from birth and the owners could therefore perceive it as "normal vision". In other words, it is "normal" to them, since it has always been so.

Next, a consideration of whether or not owners are competent to evaluate if their dog possess normal vision is done. Observations of mismatch between the owners' perception of their dogs' vision and the actual vision when tested clinically, was made by Blogg, (1970). If this observation represents the general situation, then the questionnaire answers would not represent true clinical vision.

The possibility of some answers being affected by some sort of bias is also considered. Since it is the general opinion that vision is uncompromised unless dogs are severely phenotypically affected, one could argue if this could influence some owners to answer the questions based on a prejudiced opinion of their dogs' vision.

Aside from the above mentioned factors, we must bear in mind that clinical observations of vision is not a direct expression of the actual visual function.

Histopathological changes in the retina of some CRD affected dogs, have been characterized by an attenuation of the outer nuclear layer, absence of rods and cones and a reduced number of ganglion cells (Roberts, et al., 1966b). These lesions will certainly affect retinal function and thereby in some way also vision (Narfström, 2015). It might however not be noticed since dogs have a remarkable ability to adapt to loss of vision. Unlike humans, dogs rely much more on their well-developed senses of smell and hearing to navigate (Orfri, 2007). Said in other words, dogs can have inferior vision without us noticing it.

As described, the breeding restrictions and recommendations differ substantially between affected breed. This is probably an expression of the very different prevalence's of CEA in the breeds, combined with different perceptions of the significance of the anomaly.

It would not be possible to cull all affected dogs in a breed with a very high prevalence, since it would reduce the breeding population considerable resulting in problems from inbreeding. In a breed of very low prevalence, like the Border Collie (6% Bedford, 1982b), it would be unproblematic to remove affected dogs from breeding. The Shetland Sheepdog has a roughly medium prevalence, and it is therefore possible to require at least one of the parental animals to be unaffected without contributing to inbreeding.

When considering breeding strategies for CEA, we are forced look at the entire health situation of the breed. In Shetland Sheepdogs, other potentially debilitating conditions such as HD/ED and PRA should definitely have a higher priority in regards to the breeding programs. That being said, it would be wise to work determined against slowly thinning out CEA in the population.

Conclusion

Extensive work has been put into finding a reliable genetic test for CRD, the primary phenotype of CEA. At the present time, a commercially available genetic test is marketed which tests for a deletion within the *NHEJ1* gene. The developers of the genetic test found complete compliance between the genetic test and the phenotypes of the tests subjects.

However, a previous Danish thesis study found very poor compliance when tested in a group of affected and unaffected Rough Collies.

With this study we aimed to further investigate the compliance, by genetic testing another breed known to be affected by CEA, the Shetland Sheepdog. We also sought to establish the prevalence of the genetic mutation in the breed. Moreover, we wanted to gain insight into the visional effects of CEA.

We found an allele frequency of the genetic mutation of 0,58, but since no similar studies have been made in the breed in other countries, we cannot draw a direct comparison.

Our results showed a very good compliance between the genetic test and the clinical diagnosis of CRD. Only in 5 cases of blue merle dogs were there inconsistencies. These cases might be misdiagnosed due to the difficulties associated with clinical diagnosis in dogs of this particular coat color. Based solely on our findings, it would seem that the DNA test would be an appropriate additional diagnostic tool in the Shetland Sheepdog.

However, we cannot overlook the collective evidence which questions a simple recessive mode of inheritance of CRD and thereby also the reliability of the genetic test.

In conclusion, at the present time too many uncertainties of the mode of inheritance of CRD exist and therefore should the genetic test not be used as the sole diagnostic tool. If the genetic test is used in addition of the eye examination, an overall assessment should be made.

In regards to the visional effects of CEA, we found that owners of most CEA affected dog experience their dog to possess normal vision. However, the validity of these results is questioned.

The review of literature on CEA found an almost complete lack of scientific studies on the subject. Histological studies of CEA associated retinal changes were analyzed and the lesions described were found to cause some type of decreased vision. It was later discussed how some visional deficits might be unnoticed due to the remarkable adaptability of dogs.

To conclude, scientific studies of the visional effects of CEA are most needed. Such studies would help to assess the impact of the anomaly and also aid in deciding how much effort should be spend eradicating CEA.

Perspectives

Based on the conclusion, further similar studies are needed to clarify the cause of the conflicting results in regards to compliance found by us and by Jönsson and Söderlund (2014). Such studies should be conducted in a much larger study population to give results statistical certainty.

Before commencing an extensive genetic detective work to find the cause of the inconsistencies, one could begin with reexamining the eyes of the collies which were clinically unaffected but homozygous for the NHEJ1 gene mutation.

By examining the eyes histologically post mortem or perhaps via OCT in vivo, one could detect the otherwise clinically undetectable minor lesions if present. Such studies would also reveal if some of the dogs, although seemingly unlikely, had been subjected to the "go-normal" effect. As described, the choroidal lesions are present in these dogs but masked by pigment. With OCT each of layers of the fundus can be evaluated individually and if minor hypoplasia is present, it will be identified. If no signs of affliction could be demonstrated, then a genetic source would be even more probable and the genetic test should be thoroughly reexamined.

We have discussed the likelihood of a more significant part of CEA affected dogs having some degree of visional impairment, than that of today's general perception. As it is now, there is no tangible evidence supporting this theory.

New studies investigating vision in a more measurable manner would therefore be advised. Such studies should include a large study population of dogs with different degrees of severity of CEA, and control groups of unaffected dogs from the same breeds. Several methods have been used to evaluate the visional capacity of dogs, but the author finds the work by (Gearhart, et al., 2008) especially worth looking into.

In regards to breeding restrictions and recommendations, the extensive work already done by the Health Committee of the Danish Shetland Sheepdog Club is recognized. Since the new addition to the breeding recommendations have just been implemented, it would be interesting to follow the development of the mutation allele frequency in the coming years.

It is found preferable to introduce a breeding restriction which requires eye examination to be conducted before 10 weeks of age. This would reduce the possibility of “go-normals” being used for breeding based on a false diagnosis. Additionally a breeding restriction for dogs with coloboma should be employed. Such a restriction would reduce the occurrence of the secondary changes which are known to have detrimental effect on vision.

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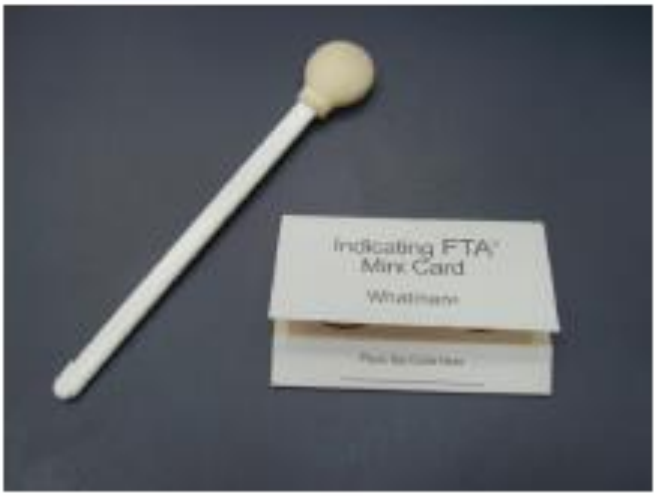
APPENDIX

Appendix A– Buccal mucosal DNA sample collection guide

VEJLEDNING
TIL OPSAMLING AF DNA
FRA MUNDSLIMHINDEN



1. Læs vejledningen inden du går i gang.

2. Tænk på følgende:

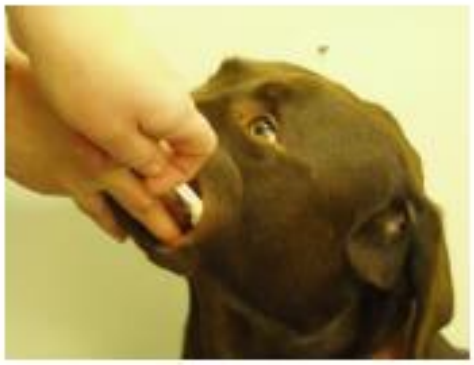
- ✓ Din hund skal ikke have spist eller drukket umiddelbart før prøvetagning.
- ✓ Undgå så vidt muligt at skumgummiet på prøvepinden kommer i kontakt med anden end hundens mundslimhinde og det lyserøde filtrerpapir på FTA-kortet.
- ✓ Når skumgummipuden har været i kontakt med FTA-kortet skal du ikke 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.

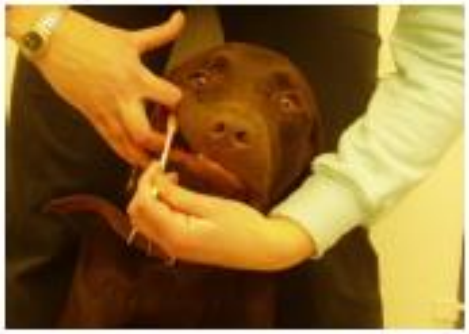
4. Skriv hundens komplette navn (inkl. kennelnavn) og stambogs-nummer på FTA-kortets forside.

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

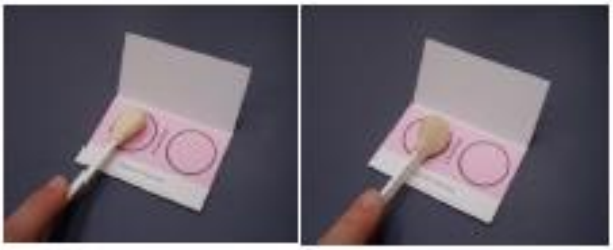
6. Adskil hundens over- og underlæbe ved hjælp af dine fingre. Få evt. en hjælper til at holde hunden hvis den er urolig. Hold prøvepinden i den anden hånd og før den ind på indersiden af kinden. Gnub skumgummiet forsigtigt mod indersiden af kinden i ca. 30 sekunder.



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.

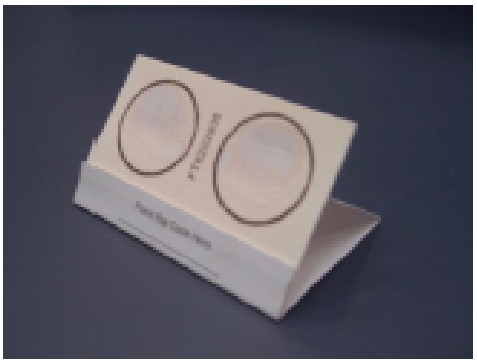


7. Nu skal materialet overføres til filtrerpapiret. Tryk skumgummipuden mod papiret indenfor den ene sorte cirkel og vip puden fra side til side med en rullende bevægelse. **GNUB IKKE - papiret går nemt i stykker.** Vend skumgummipuden og overfør resten af materialet til den anden cirkel



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 B – Whatman® FTA card manual

Whatman®
Schleicher & Schuell

FTA® Cards

DESCRIPTION: FTA cards are designed for room temperature collection, shipment, archiving, and purification of nucleic acids from a wide variety of biological samples for PCR analysis. These include (but are not limited to) blood, buccal cells, tissue, cultured cells, microorganisms, and plant tissue. FTA Cards are impregnated with a patented chemical formula that lyses cell membranes and denatures proteins upon contact. Nucleic acids are immobilized and protected from UV damage and microbial and fungal attack. Infectious pathogens in samples applied to FTA Cards are rendered inactive upon contact. FTA Cards are available in several formats such as the Classic Card, Mini Card, Micro Card, and Gene Card. Indicating FTA Cards turn from pink to white upon sample application and are recommended for colorless samples. To use FTA Cards, simply apply sample (liquid or pressed tissue), air dry at room temperature, then remove a small disc (1.2 mm or 2.0 mm, depending on application). The disc is then washed and used in PCR-based analysis.

PRECAUTIONS:

HANDLING: Always wear gloves to avoid contamination of FTA Cards. Follow universal precautions when handling biological specimens.

STORAGE: Store unused cards in original packaging in a cool, dry, clean environment. After applying samples, allow them to dry, then store at room temperature in a dry environment.

INSTRUCTIONS:

Application of Blood Samples (fresh whole blood, or with the anticoagulants: EDTA, sodium citrate, ACD,or heparin):

1. Label the FTA card with the appropriate sample identification.
2. Drop the blood (< 125 µL per 1 inch circle, < 75 µL per ¾ inch circle) onto the card in a concentric circular motion within the printed circle area. Avoid "puddling" of the liquid sample, as it will overload the chemicals on the card. Also, do not rub or smear the blood onto the card.
3. Samples applied to FTA cards are ready for immediate room temperature storage. Note: If samples are to be processed shortly after application on the FTA card, allow the sample to dry for one hour at room temperature prior to punching. Do not heat to shorten the drying period.
4. Dried blood spots will appear darker than freshly spotted ones.
5. The sample is now ready for downstream processing or archive (see reverse).

Collection and application of buccal cell samples:

1. Place the FTA card (Indicating FTA is recommended) on a clean, dry, flat surface. Label the FTA Card with appropriate sample identification.
2. Remove one Foam Tipped Applicator (Whatman Cat. No. WB100032) from the protective packaging according to instructions.
3. Hold the plastic handle of the Applicator, place the foam tip in the mouth and rub one side of the foam tip on the inside of the cheek for 30 sec. Repeat using the opposite side of the foam tip for the other cheek. Run the foam tip along the gum-line and fold of the cheek and under the tongue, soaking up as much saliva as possible. Remove the Applicator from the mouth.
4. Lift the paper cover of the Indicating FTA Card to expose the pink sample area. Press the flat surface of the foam applicator tip within the sample circle area. Without lifting the foam tip from the card, squeeze the tip using a side-to-side rocking motion (90° in each direction) 3 times to completely saturate the sample area. Turn the Applicator over and repeat with the other side of the foam tip within the same circle. The sample area will turn white indicating the location of sample.
5. If not using Indicating FTA Cards, circle the area of the sample location with a ballpoint pen or pencil.
6. Discard the Applicator according to laboratory procedure. Do not place the foam swab into the mouth after it has touched the FTA card.
7. If buccal cells are to be applied to more than one FTA circle area, use a new Applicator and repeat steps 1 - 6.
8. Samples applied to FTA cards are ready for immediate room temperature storage. Note: If samples are to be processed shortly after application on the FTA card, allow the sample to dry for one hour at room temperature prior to punching. Do not heat to shorten the drying period.
9. The sample is now ready for downstream processing or archive (see reverse).

Application of Tissue/Cell culture samples:

1. Tissue culture cells should be applied to FTA Cards at a concentration of > 100 cells/µl for DNA analysis and >1000 cells/µl for RNA analysis in media, trypsin, or PBS buffer. Approximately 65µl of sample will fill a 1 inch printed circle on an FTA Card.
2. Samples applied to FTA cards are ready for immediate room temperature storage. Note: If samples are to be processed shortly after application on the FTA card, allow the sample to dry for one hour at room temperature prior to punching. Do not heat to shorten the drying period.
3. The sample is now ready for downstream processing or archive (see reverse).

Application of Plant samples:

Direct leaf press:
1. Place leaf material directly onto the FTA card. Lay a piece of parafilm over the leaf.
2. Apply moderate pounding/pressure to the leaf area with a blunt object such as a tack hammer or pestle.
3. When the extract is drawn through to the back of the FTA card the collection process is complete.
4. Samples applied to FTA cards are ready for immediate room temperature storage. Note: If samples are to be processed shortly after application on the FTA card, allow the sample to dry for one hour at room temperature prior to punching. Do not heat to shorten the drying period.
5. The sample is now ready for downstream processing or archive.

FTA® Cards

Plant Tissue Homogenate:

1. Use about 10-20 mg of plant tissue for the homogenate.
2. Add PBS buffer to plant tissue using an estimated ratio of 5 parts PBS buffer to 1 part plant tissue. Grind with a pestle until it is apparent that some plant tissue is homogenized. The homogenate does not have to be smooth in consistency.
3. Using a pipette, apply about 25µl of plant homogenate to each circle on an FTA card.
4. Samples applied to FTA cards are ready for immediate room temperature storage. Note: If samples are to be processed shortly after application on the FTA card, allow the sample to dry for one hour at room temperature prior to punching. Do not heat to shorten the drying period.
5. The sample is now ready for downstream processing or archive (see below).

Application of Bacterial Samples (for bacterial genomic DNA)

Bacterial colonies:

1. Pick one colony from agar, and suspend in 5-10 µl of bacterial culture medium, PBS, or TE^{-1} buffer (10 mMTris-HCl, 0.1 mM EDTA, pH 8.0).
2. Apply 5-10 µl of bacterial suspension to FTA (Indicating FTA Cards are recommended). If applied to non-Indicating FTA cards, circle the area of application with a ballpoint pen or pencil.
3. Samples applied to FTA cards are ready for immediate room temperature storage. Note: If samples are to be processed shortly after application on the FTA card, allow the sample to dry for one hour at room temperature prior to punching. Do not heat to shorten the drying period.
4. The sample is now ready for downstream processing or archive (see below).

Overnight Bacterial Cultures:

1. Take about 65 µl of overnight culture and apply to FTA (Indicating FTA Card is recommended). If applying to non-Indicating FTA, circle the area of application with a ballpoint pen or pencil.
2. Samples applied to FTA cards are ready for immediate room temperature storage. Note: If samples are to be processed shortly after application on the FTA card, allow the sample to dry for one hour at room temperature prior to punching. Do not heat to shorten the drying period.
3. The sample is now ready for downstream processing or archive (see below). .

Archiving of samples on FTA Cards:

Biological samples applied to FTA cards should be archived at room temperature in a Multi-Barrier Pouch (Whatman Cat. Nos: WB100036 or WB100037) with a desiccant (WB100003) or stored in a humidity-controlled, cool, dry environment. Samples for RNA analysis should be stored at -20°C or -70°C for long term storage.

Preparation of Sample DNA for Downstream Analysis:

1. Take a sample disc from the desired sample spot using a coring device. For blood samples and bacterial genomic DNA samples, a 1.2 mm disc is recommended. For all other sample types, use a 2.0 mm disc. Place sample disc in a PCR amplification tube.
2. Add 200 µl of FTA Purification Reagent (Whatman Cat. No.: WB120204) to PCR tube.
3. Incubate for 5 minutes at room temperature, (the tube may be given moderate manual mixing if desired)
4. Remove and discard all spent FTA Purification Reagent using a pipette.
5. Repeat steps 2-4 twice, for a total of three washes with FTA Purification Reagent*.
6. Add 200 µl of TE^{-1} Buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0) to PCR tube.
7. Incubate for 5 minutes at room temperature.
8. Remove and discard all spent TE^{-1} Buffer with a pipette.
9. Repeat steps 6-8 once for a total of two washes with TE^{-1} Buffer.
10. Allow disc to dry at room temperature for about one hour, or heat assist the drying of disc at 56°C for 10minutes. The FTA disc is now ready for PCR.

* If purifying sample disc from a plant source or bacterial culture, only two washes with the FTA Purification Reagent are necessary.

Downstream PCR Applications:

The washed and dried disc is now ready for PCR analysis. Assuming a 25ul reaction, we recommend a 1.2 mm disc for blood and 2.0mm disc for buccal and bacterial samples. The disc is included in the PCR reaction. No alterations in the PCR reaction mix or cycling conditions are required.

FOR ADDITIONAL PROTOCOL INFORMATION, PLEASE REFER TO THE FTA APPLICATIONS GUIDE, CAT.NO. WB120067 OR VISIT WWW.WHATMAN.COM.

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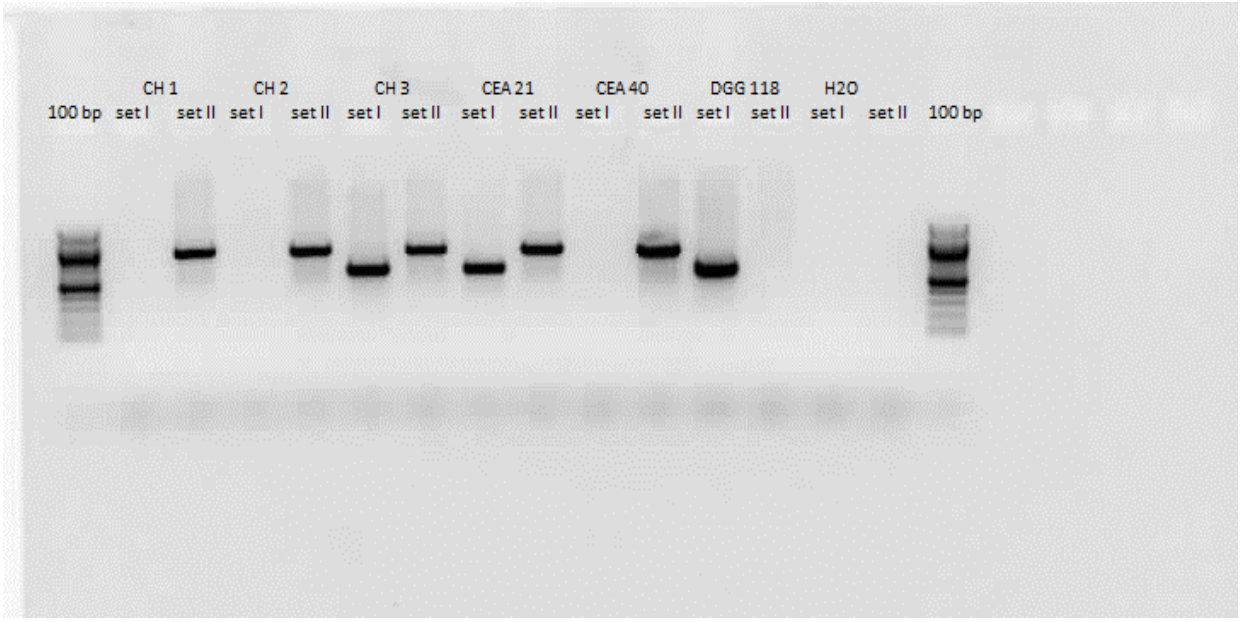
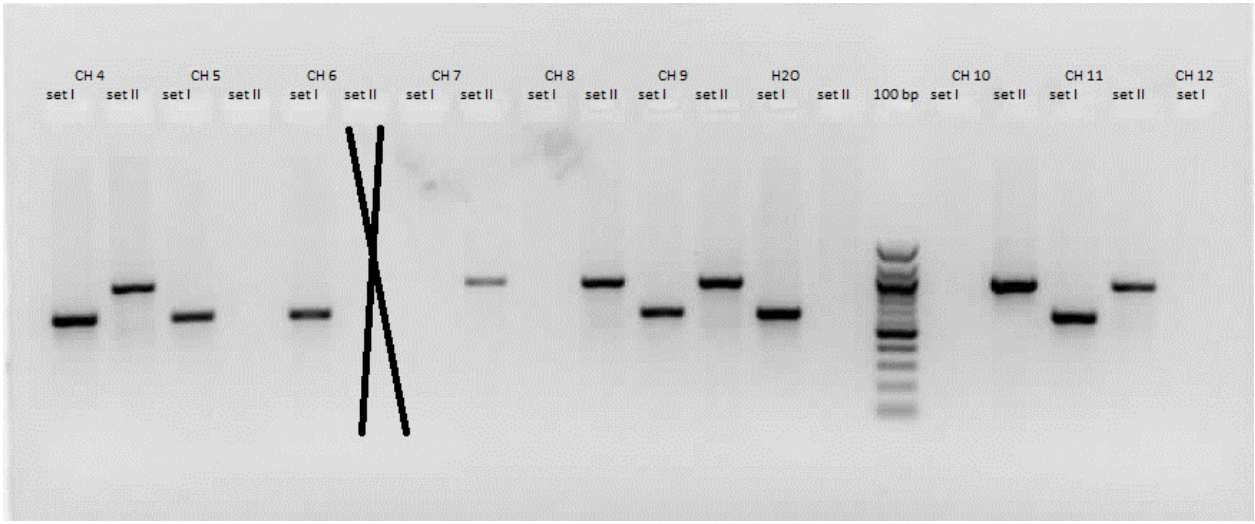
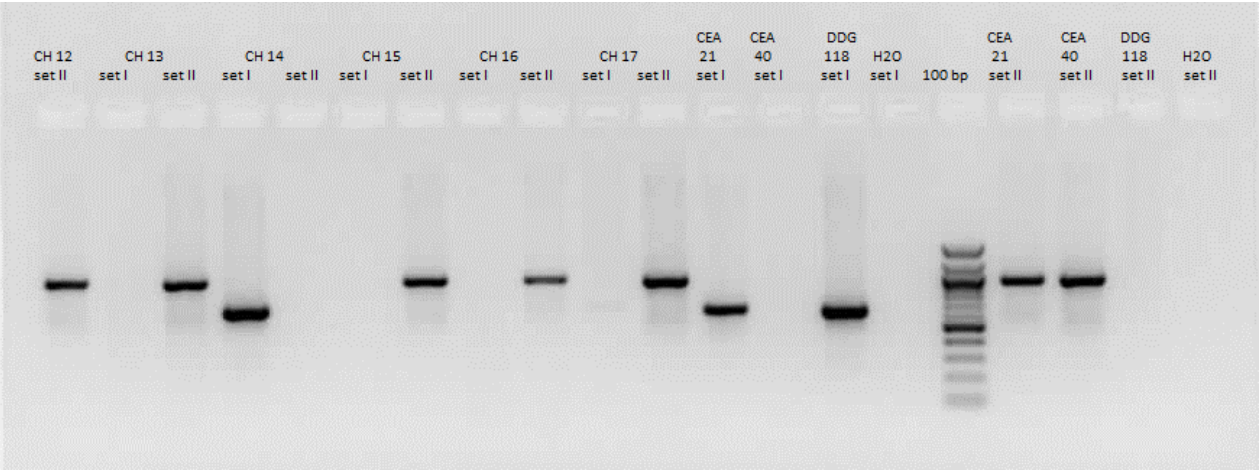
Telephone: +65 6534 0138 • Fax: +65 6534 2166 • E-mail: wap@whatman.com

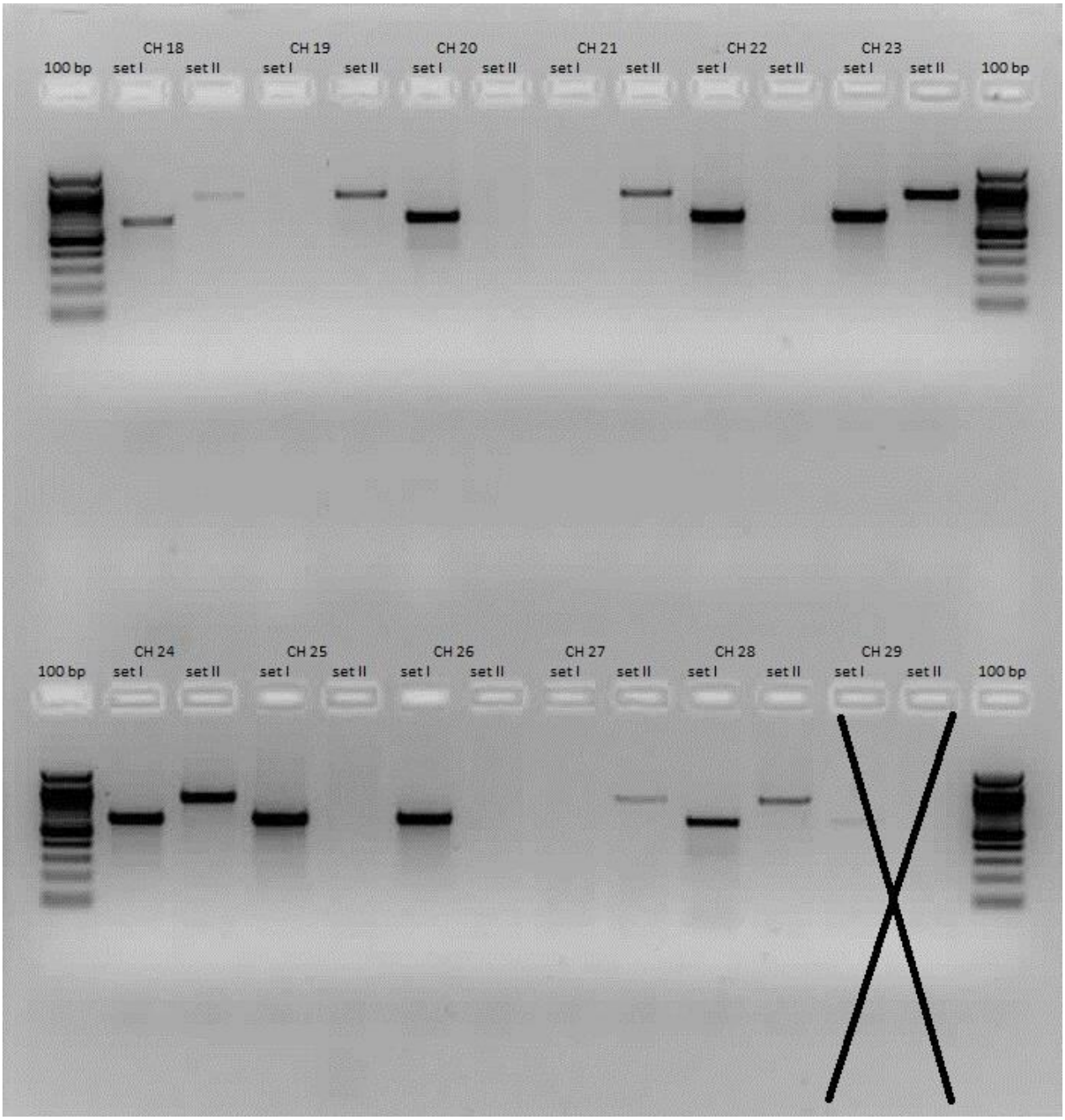
Appendix C - Electrophoretograms

Electrophoretograms demonstrating the PCR results. Samples in lanes marked by an X was redone and results can be found in the final electrophoretogram.






CH 12 set II
CH 13 set I
CH 13 set II
CH 14 set I
CH 14 set II
CH 15 set I
CH 15 set II
CH 16 set I
CH 16 set II
CH 17 set I
CH 17 set II
CEA 21 set I
CEA 40 set I
DDG 118 set I
H2O set I
100 bp
CEA 21 set II
CEA 40 set II
DDG 118 set II
H2O set II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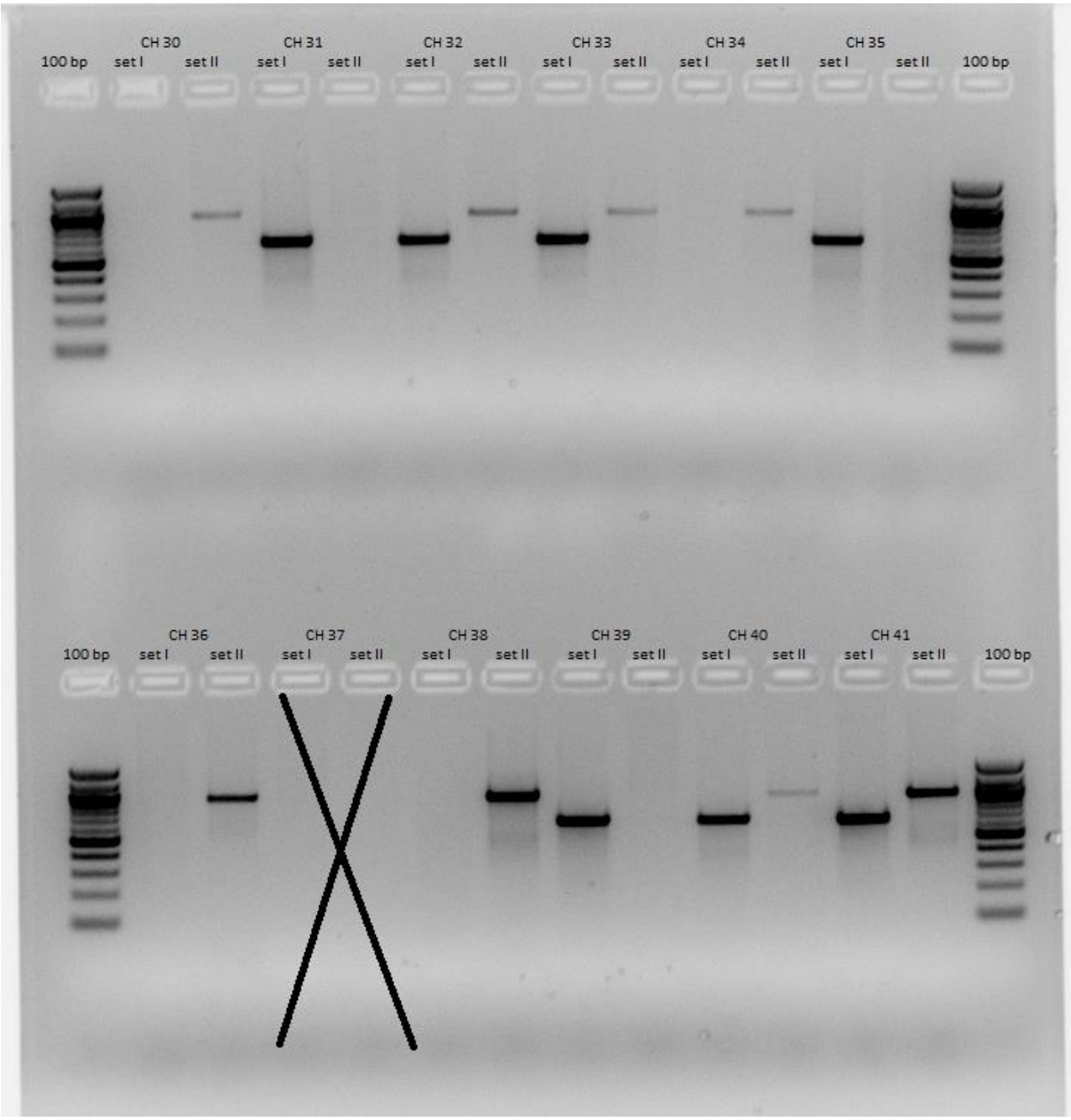
CH 18 set I
CH 18 set II
CH 19 set I
CH 19 set II
CH 20 set I
CH 20 set II
CH 21 set I
CH 21 set II
CH 22 set I
CH 22 set II
CH 23 set I
CH 23 set II
100 bp
100 bp
CH 24 set I
CH 24 set II
CH 25 set I
CH 25 set II
CH 26 set I
CH 26 set II
CH 27 set I
CH 27 set II
CH 28 set I
CH 28 set II
CH 29 set I
CH 29 set II
100 bp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CH 30
set I
set II
CH 31
set I
set II
CH 32
set I
set II
CH 33
set I
set II
CH 34
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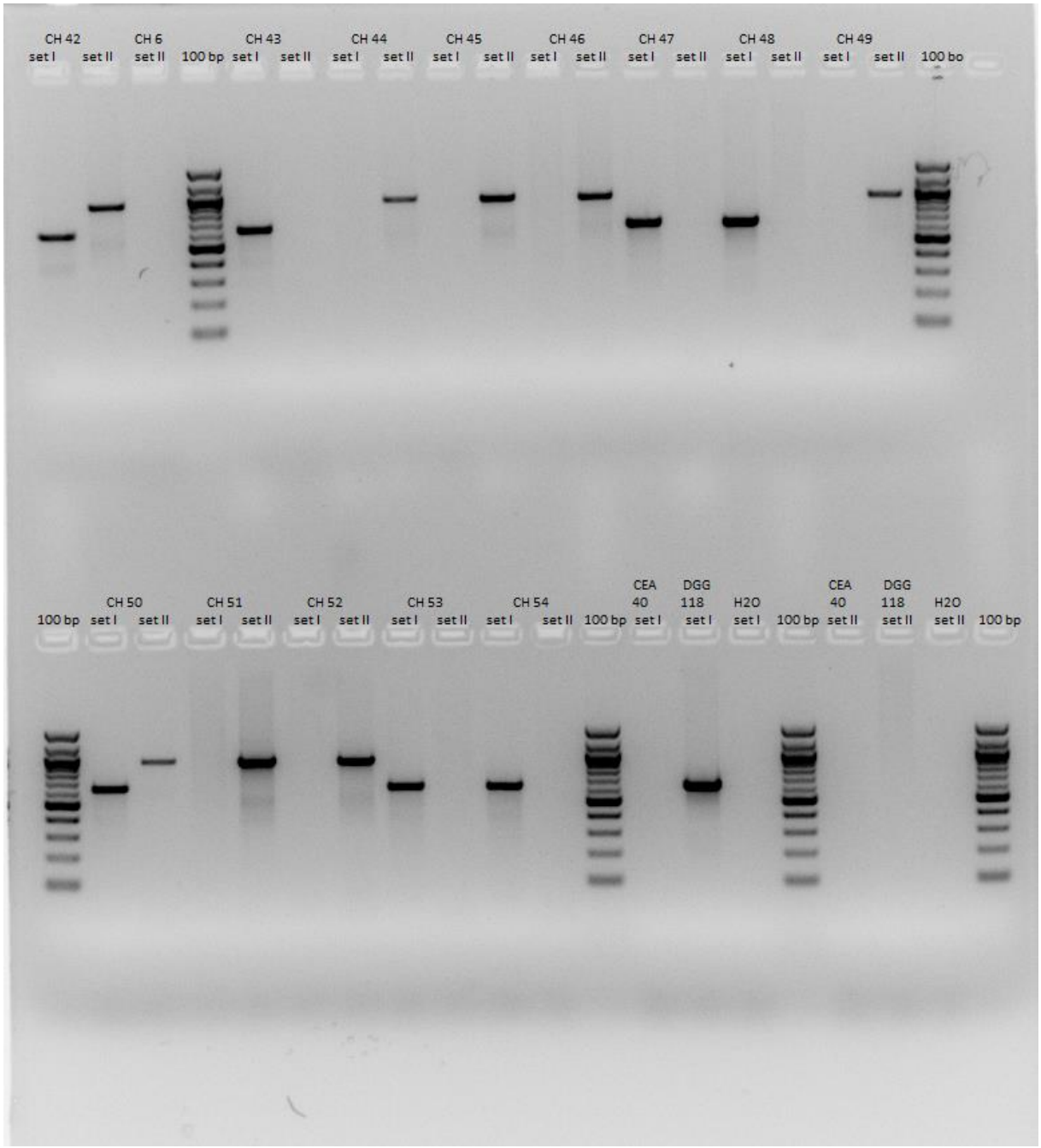
set II
CH 35
set I
set II
100 bp
100 bp
CH 36
set I
set II
CH 37
set I
set II
CH 38
set I
set II
CH 39
set I
set II
CH 40
set I
set II
CH 41
set I
set II
100 bp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set I
set II
CH 6
set II
100 bp
CH 43
set I
set II
CH 44
set I
set II
CH 45
set I
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set I
set II
CH 47
set I
set II
CH 48
set I
set II
CH 49
set I
set II
100 bo
100 bp
CH 50
set I
set II
CH 51
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set II
CH 52
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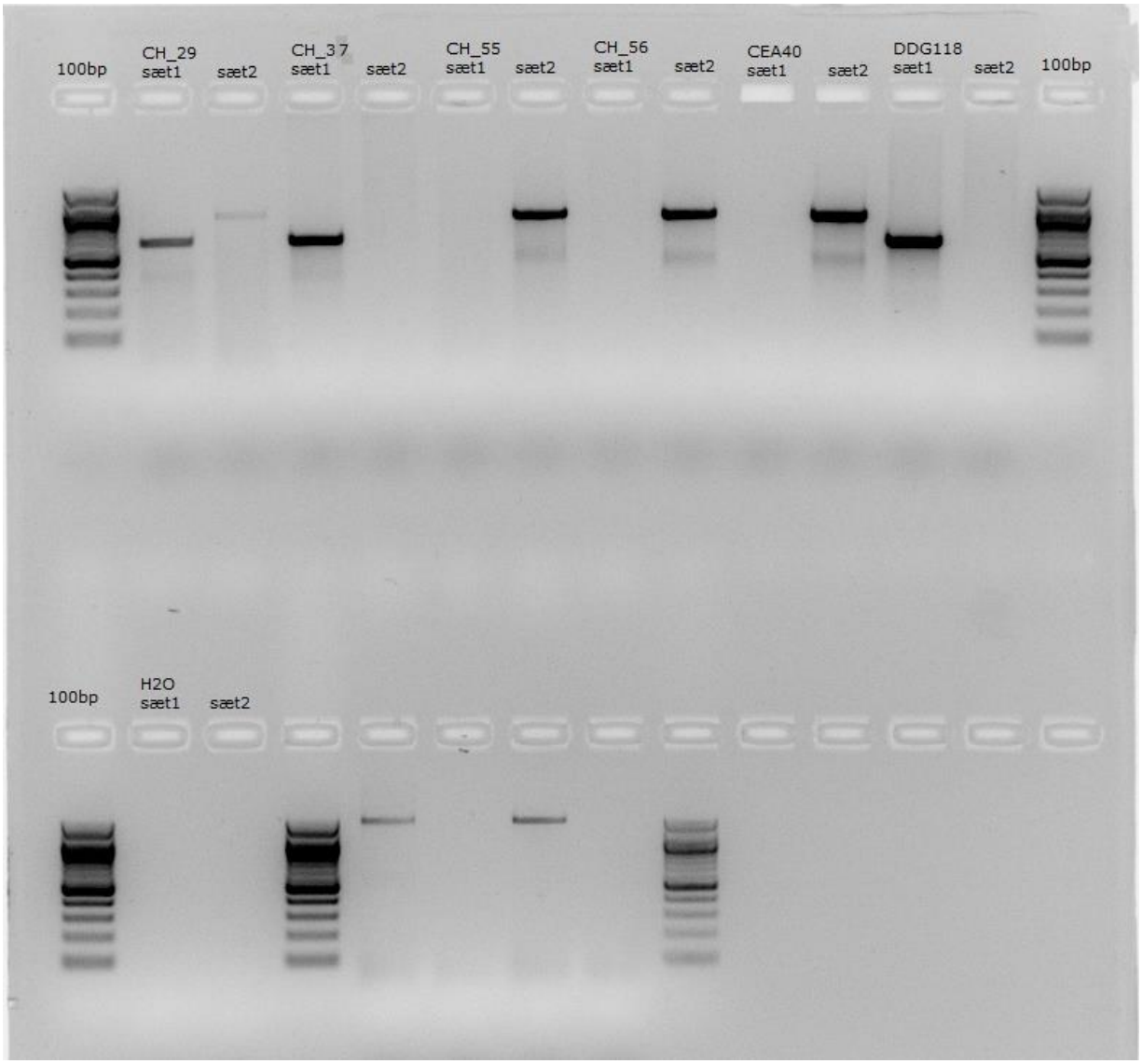
set II
CH 53
set I
set II
CH 54
set I
set II
100 bp
CEA 40
set I
DGG 118
set I
H2O
set I
100 bp
CEA 40
set II
DGG 118
set II
H2O
set II
100 bp

X


100bp
CH_29 sæt1
sæt2
CH_37 sæt1
sæt2
CH_55 sæt1
sæt2
CH_56 sæt1
sæt2
CEA40 sæt1
sæt2
DDG118 sæt1
sæt2
100bp
100bp
H2O sæt1
sæt2

Appendix D – Results of genotyping

Clinical diagnosis, genotyping results and coat color of participating dogs.

Lab ID	Clinical diagnosis	DNA-test	Coat color
SS_CH_001	CRD	crd/crd	Zobel/White
SS_CH_002	CRD	crd/crd	Zobel/White
SS_CH_003	Nothing demonstrated	CRD/crd	Blue Merle
SS_CH_004	Nothing demonstrated	CRD/crd	Zobel/White
SS_CH_005	Nothing demonstrated	CRD/CRD	Black and White
SS_CH_006	Nothing demonstrated	CRD/CRD	Black and White
SS_CH_007	CRD	crd/crd	Zobel/White
SS_CH_008	CRD	crd/crd	Tricolour
SS_CH_009	Nothing demonstrated	CRD/crd	Zobel/White
SS_CH_010	CRD	crd/crd	Tricolour
SS_CH_011	Nothing demonstrated	CRD/crd	Tricolour
SS_CH_012	CRD , small coloboma unilaterally	crd/crd	Tricolour
SS_CH_013	CRD, coloboma	crd/crd	Tricolour
SS_CH_014	Nothing demonstrated	CRD/CRD	Zobel/White
SS_CH_015	CRD	crd/crd	Tricolour
SS_CH_016	CRD	crd/crd	Zobel/White
SS_CH_017	CRD	crd/crd	Zobel/White
SS_CH_018	CRD	CRD/crd	Blue Merle
SS_CH_019	CRD, vitreal hemorrhage unilaterally	crd/crd	Zobel/White
SS_CH_020	CRD	CRD/CRD	Blue Merle
SS_CH_021	CRD	crd/crd	Blue Merle
SS_CH_022	Nothing demonstrated	CRD/CRD	Blue Merle
SS_CH_023	Nothing demonstrated	CRD/crd	Blue Merle
SS_CH_024	Nothing demonstrated	CRD/crd	Zobel/White
SS_CH_025	Nothing demonstrated	CRD/CRD	Blue Merle
SS_CH_026	Nothing demonstrated	CRD/CRD	Zobel/White
SS_CH_027	CRD	crd/crd	Zobel/White
SS_CH_028	Nothing demonstrated	CRD/crd	Zobel/White
SS_CH_029	Nothing demonstrated	CRD/crd	Tricolour
SS_CH_030	CRD	crd/crd	Tricolour
SS_CH_031	CRD	CRD/CRD	Blue Merle
SS_CH_032	Nothing demonstrated	CRD/crd	Tricolour
SS_CH_033	Nothing demonstrated	CRD/crd	Zobel/White
SS_CH_034	CRD	crd/crd	Blue Merle
SS_CH_035	Nothing demonstrated	CRD/CRD	Zobel/White

SS_CH_036	CRD	crd/crd	Zobel/White
SS_CH_037	Nothing demonstrated	CRD/CRD	Blue Merle
SS_CH_038	CRD	crd/crd	Blue Merle
SS_CH_039	Nothing demonstrated	CRD/CRD	Blue Merle
SS_CH_040	CRD	CRD/crd	Blue Merle
SS_CH_041	Nothing demonstated	CRD/crd	Tricolour
SS_CH_042	Nothing demonstrated	CRD/crd	Zobel/White
SS_CH_043	Nothing demonstrated	CRD/CRD	Tricolour
SS_CH_044	CRD	crd/crd	Zobel/White
SS_CH_045	CRD	crd/crd	Zobel/White
SS_CH_046	Nothing demonstrated	crd/crd	Blue Merle
SS_CH_047	Nothing demonstrated	CRD/CRD	Zobel/White
SS_CH_048	Nothing demonstrated	CRD/CRD	Zobel/White
SS_CH_049	CRD, Retinal detachment on right eye	crd/crd	Zobel/White
SS_CH_050	Nothing demonstrated	CRD/crd	Zobel/White
SS_CH_051	CRD	crd/crd	Zobel/White
SS_CH_052	CRD	crd/crd	Zobel/White
SS_CH_053	Nothing demonstrated	CRD/CRD	Blue Merle
SS_CH_054	Nothing demonstrated	CRD/CRD	Tricolour
SS_CH_055	CRD	crd/crd	Zobel/White
SS_CH_056	CRD	crd/crd	Zobel/White