Comprehensive study on multiple genetic disorders in pure breed dogs

（純血種犬の多種遺伝子病に関する包括的研究）

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ABSTRACT

In veterinary medicine, it is necessary to prevent and control genetic disorders in pure dog breeds in order to preserve the quality of these companion animals. The entire canine genome sequence was completed approximately 10 years ago and more than 150 disease-causing or associated mutations have been found in dogs. This advancement in canine genetics has contributed to many preventive measures against genetic disorders in pure breed dogs. However, a comprehensive study of multiple genetic disorders has not yet been performed in pure breed dogs. The objectives of my thesis are to develop novel molecular genotyping methods for canine multiple genetic disorders and to collect clinical and epidemiological data using these methods. Its aim is to prevent and/or control genetic disorders in pure breed dogs.

The Border Collie breed was chosen as a target in the present study. In this breed, 6 kinds of molecularly defined genetic defects were reported: neuronal ceroid lipofuscinosis (NCL), trapped neutrophil syndrome (TNS), a 4-bp deletion in the canine *MDR1*/*ABCB1* gene associated with ivermectin toxicosis (IT), Collie eye anomaly (CEA), selective cobalamin malabsorption (SCM), degenerative myelopathy (DM), and a congenital ocular and auditory abnormality associated with a merle hair coat (MER). The Border Collie breed also has a single nucleotide polymorphism in the *MDR1*/*ABCB1* gene associated with phenobarbital resistant idiopathic epilepsy (PRE). These 8 genetic disorders were investigated in this study. In this study, several genotyping methods were newly developed for NCL, TNS, IT, SCM, and PRE. Previous genotyping methods were used for CEA, DM, and MER. These genotyping assays included polymerase chain reaction (PCR)-length polymorphism assay, PCR-restriction fragment length polymorphism assay, PCR-primer-induced restriction

analysis assay, mutagenically separated-PCR assay, and SYBR Green- and TaqMan minor groove binder probe-based real-time PCR assays. These genotyping assays were used for diagnosis of affected animals and epidemiological survey in the population of 478 Border Collies randomly collected from all over Japan.

Regarding NCL, the number of affected Border Collies was surveyed, and their clinical characteristics were analyzed. In 4 kennels with affected dogs, the dogs were genotyped. The genetic relationships of all affected dogs and carriers identified were analyzed. Regarding TNS, clinical and clinico-pathologic findings in a Border Collie that was molecularly diagnosed as TNS for the first time in Japan were analyzed. CEA was examined molecularly and epidemiologically in Hokkaido dogs. In addition, the genetic relationship between CEA and IT was investigated using 9 canine breeds. Finally, a simulation analysis for prevention of multiple genetic disorders in pure breed dogs was performed on the basis of the allele frequencies of 7 genetic disorders reported in Border Collies.

All assays developed for genotyping in this study were available to discriminate all 3 genotypes, i.e., wild-type, carrier and affected animals. Of all assays, real-time PCR methods were the most suitable for large-scale examination because of its high throughput. The genotyping survey among 478 Border Collies demonstrated that mutant allele frequencies of NCL, TNS, IT, CEA, SCM, DM, and MER were 3.5%, 5.7%, 0.2%, 14.5%, 1.5%, 0.7%, and 1.5%, respectively. The epidemiological survey for NCL revealed 28 affected dogs, but there was a decreasing trend at the end of the study period. The clinical characteristics of these affected dogs were updated in detail. The genotyping survey demonstrated a high mutant allele frequency (34.8%) in the 4 examined kennels. The pedigree analysis demonstrated that

all affected dogs and carriers in Japan are related to some presumptive carriers imported from Oceania and having a common ancestor. The current high prevalence in Japan might be due to an overuse of these carriers by breeders without any knowledge of the disease. TNS was first molecularly diagnosed in Japan in a dog with characteristic clinical and clinico-pathologic findings. A Hokkaido dog affected ophthalmologically with CEA was proven to possess the CEA-associated mutation. The genotyping survey and pedigree analysis demonstrated that the allelic frequency of the CEA-associated mutation is very high (64.7%) in Hokkaido dogs. However, there was no apparent genetic relationship between CEA and IT based on the data collected from 9 canine breeds including Hokkaido dogs. The simulation analysis of prevention of multiple genetic disorders in Border Collies demonstrated the following issues. In the 7 genetic disorders except for PRE, the ratio of animals having 1, 2, and 3 mutant alleles was 35.2%, 8.4%, and 0.4%, respectively. There was no animal that had more than 4 different mutant alleles. The resulting ratio of animals without all 7 mutant alleles was still high (56.1%). This finding suggested that the preventive control using dogs free from the 6 genetic disorders can be carried out without changing the breed characteristics of Border Collies. The allele frequency of PRE was very high (24.9%) in Border Collies, but this allele should not be removed because it may be a polymorphism.

In conclusion, genotyping assays and clinical and epidemiological findings described in the present study will contribute to the establishment of canine breeds free from multiple genetic disorders in the future as well as provide novel clinical information and diagnostic methods for veterinary practitioners and researchers.

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FREQUENTLY USED ABBREVIATION

A	-	adenine
bp	-	base pair
C	-	cytosine
CEA	-	collie eye anomaly
DNA	-	deoxyribonucleic acid
DM	-	degenerative myelopathy
EDTA	-	Tris–ethylenediamine tetra-acetic acid
FTA	-	Flinders Technology Associates
PRE	-	phenobarbital resistant idiopathic epilepsy
G	-	guanine
LP	-	length polymorphism
NCL	-	neuronal ceroid lipofuscinosis
MER	-	congenital eye disease and deafness associated with merle coat
IT	-	a 4-bp deletion in the canine *MDR1*/*ABCB1* gene associated with ivermectin toxicosis
MGB	-	minor groove binder
MS-PCR	-	mutagenically separated-polymerase chain reaction
PCR	-	polymerase chain reaction
RFLP	-	restriction fragment length polymorphism
PIRA	-	polymerase chain reaction-primer-induced restriction analysis
P-gp	-	P-glycoprotein
SCM	-	selective cobalamin malabsorption
SINE	-	short interspersed element
SNP	-	single nucleotide polymorphism
T	-	thymine
TNS	-	Trapped neutrophil syndrome
*T*m	-	melting temperature

CHAPTER 1

Rapid diagnostic assays for neuronal ceroid lipofuscinosis in Border Collies

The above-titled work originally appeared in "*Journal of Veterinary Diagnostic Investigation* (Mizukami et al., 2011)" as: ***Novel rapid genotyping assays for neuronal ceroid lipofuscinosis in Border Collie dogs and high frequency of the mutant allele in Japan*** authored by:

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1.1. ABSTRACT

Neuronal ceroid lipofuscinosis (NCL) constitutes a group of recessively inherited lysosomal storage diseases that primarily affect neuronal cells. Such diseases share certain clinical and pathologic features in human beings and animals. Neuronal ceroid lipofuscinosis in Border Collie dogs was first detected in Australia in the 1980s, and the pathogenic mutation was shown to be a nonsense mutation (c.619C>T) in exon 4 in canine *CLN5* gene. In the present study, novel rapid genotyping assays including polymerase chain reaction (PCR)–restriction fragment length polymorphism, PCR primer–induced restriction analysis, mutagenically separated PCR, and real-time PCR with TaqMan minor groove binder probes were developed. The utility of microchip electrophoresis was also evaluated. Furthermore, a genotyping survey was carried out in a population of Border Collies in Japan using these assays to determine the current allele frequency in Japan, providing information to control and prevent this disease in the next stage. All assays developed in the current study are available to discriminate these genotypes, and microchip electrophoresis showed a timesaving advantage over agarose gel electrophoresis. Of all assays, real-time PCR was the most suitable for large-scale examination because of its high throughput. The genotyping survey demonstrated that the carrier frequency was 7.0%. This finding suggested that the mutant allele frequency of NCL in Border Collies is high enough in Japan that measures to control and prevent the disease would be warranted. The genotyping assays developed in the present study could contribute to the prevention of NCL in Border Collies.

1.2. INTRODUCTION

Neuronal ceroid lipofuscinosis (NCL) constitutes a group of recessively inherited lysosomal storage diseases that primarily affect neuronal cells. Such diseases share certain clinical and pathologic features in human beings and animals (Jalanko and Braulke, 2009). Typical clinical signs of these progressive neurodegenerative diseases include behavioral abnormality, sleep problems, mental retardation, dementia, seizure, motor abnormality such as ataxia, and/or in most cases visual problems leading to blindness (Jalanko and Braulke, 2009; Jolly and Palmer, 1995). In veterinary medicine, NCL has been described in several domestic species and occurs most commonly in the dog (Jolly et al., 1994).

NCL is characterized by massive accumulations of autofluorescent lysosomal storage bodies in the lysosomes of a variety of cell types, particularly neurons and retinal cells (Jalanko and Braulke, 2009). Accumulation of subunit c of mitochondrial adenosine triphosphate synthase has been demonstrated as the dominant storage protein in most human forms of NCL, as well as in cattle, horses, and several breeds of sheep and dog (Jolly and Palmer, 1995; Url et al., 2001). In contrast, sphingolipid activator proteins A and D are the major protein components of storage bodies in a few human forms of NCL (Nijssen et al., 2003; Tyynelä et al., 1993), in affected Swedish Landrace sheep (Tyynelä et al., 2000), Miniature Schnauzer dogs (Palmer et al., 1997), and Polish Lowland Sheepdogs (Narfström et al., 2007). However, the mechanisms of storage and neurodegeneration in NCL remain to be explained.

In canine NCL, mutations in several genes responsible for the disease were reported in English Setters (*CLN8*) (Katz et al., 2005), Border Collies (*CLN5*) (Melville et al., 2005),

Bulldogs (*CTSD*) (Awano et al., 2006b), Miniature longhaired Dachshunds (*TPP1*) (Awano et al., 2006a), Miniature Dachshunds (*PPT1*) (Sanders et al., 2010), American Staffordshire Terriers (*ARSG*) (Abitbol et al., 2011), Australian Shepherds (*CLN6*) (Katz et al., 2011), and Tibetan Terriers (*ATP13A2*) (Farias et al., 2011). Thus, once a mutation is identified in a breed or family, rather simple procedures can be used for a rapid genetic diagnosis and genotype screening leading to control and eradication of the disease.

NCL in Border Collies was first detected in Australia in the 1980s (Franks et al., 1999; Studdert and Mitten, 1991; Taylor and Farrow, 1988, 1992). A diagnosis of the first case in Japan was made in 2002 (Koie et al., 2004). In 2005, the pathogenic mutation was demonstrated to be a nonsense mutation (c.619C>T) in exon 4 in the canine *CLN5* gene (Melville et al., 2005), which enabled the development of rapid and simple genetic tests. Therefore, allele frequency should be determined accurately using these newly developed assays to estimate the requirements for preventing this disease in the next stage.

Polymerase chain reaction (PCR)-based genetic assays are very important for the prevention of a single gene disorder because these assays can detect not only affected individuals but also heterozygous carriers. In the original report regarding the pathogenic mutation of NCL in Border Collies, a PCR-restriction fragment length polymorphism (RFLP) assay was shown as a genotyping test (conventional PCR-RFLP assay), but it seems difficult to judge the genotypes because of complex fragmentation after digestion by restriction endonuclease (Melville et al., 2005). Therefore, the present study developed and evaluated novel rapid and simple genotyping assays including improved PCR-RFLP, PCR primer–induced restriction analysis (PIRA), mutagenically separated (MS)-PCR, and

real-time PCR with TaqMan minor groove binder (MGB) probes for NCL in Border Collies. The utility of the microchip electrophoresis was also evaluated in each PCR-based assay. Furthermore, genotype screening was carried out in a population of Border Collies in Japan using these assays to determine the current allele frequency in Japan and provide information to control and prevent this disease in the next stage.

1.3. MATERIALS AND METHODS

Samples and treatment

In the present study, control samples for each genotype were obtained from an affected Border Collie that had been diagnosed by histopathologic examination, a heterozygous carrier that was the sire of the affected dog and an unaffected Beagle. The genotypes were confirmed using direct sequence analysis. DNA templates for each genetic test were prepared using whole blood spotted onto FTA card (FTA classic card, Whatman International Ltd, Piscataway, NJ.) or saliva spotted onto indicating FTA card (Indicating FTA Classic Card, Whatman International Ltd, Piscataway, NJ.), which had been stored at approximately 4ºC until used. For PCR-RFLP, PCR-PIRA, and MS-PCR, a 1.2 mm in diameter disc punched out of the FTA card using a hole punch (Harris Uni-Core Punch (size 1.2 mm), Whatman International Ltd, Piscataway, NJ.) was used for a DNA template after treatment as follows. The disc was placed into a 0.2-ml PCR tube. The disc on the tube bottom was washed three times for 5 min each with 100 μl of washing solution (FTA Purification Reagent, Whatman International Ltd, Piscataway, NJ.) at room temperature, rinsed twice for 5 min each with 200 μl of Tris–ethylenediamine tetra-acetic acid (EDTA) buffer (pH 8.0) (10×TE Powder (pH 8.0), Wako Pure Chemical Industries Ltd., Osaka, Japan.) and dried at 60ºC for 10 min. The treated disc was used directly as a template in all PCR tests except real-time PCR.

Conventional and improved PCR-RFLP methods

Each PCR test was carried out targeting a sequence around the mutation (c.619C>T) in exon 4 of the canine *CLN5* gene with primers shown in Table 1. The conventional PCR-RFLP assay reported previously (Melville et al., 2005) was carried out with forward (cRFLP-F) and reverse (cRFLP-R) primers in a 20 μl reaction mixture containing 10 μl of 2× PCR master mix (GoTaq Hot Start Green Master Mix, Promega Corp., Madison, WI.), 12.5 pmol of primers, and the treated disc of FTA card as a template. After the first denaturation at 95ºC for 12 min, 40 cycles of amplification were carried out, at a denaturing temperature of 95ºC for 30 sec, an annealing temperature of 59ºC for 1 min, and an extension temperature of 72ºC for 1 min. Extension during the last cycle was carried out at 72ºC for 6 min. The product of the conventional PCR-RFLP was digested with the restriction enzyme *Mse*I (New England Biolabs, Ipswich, MA.) at 37ºC for 90 min in a 10 μl reaction mixture containing 7 μl of the product, 10 U of *Mse*I, 1 μl of 10× restriction enzyme buffer (10× NE Buffer, New England Biolabs, Ipswich, MA.), and 0.1 μl of 100× bovine serum albumin (100x BSA, New England Biolabs, Ipswich, MA.) included by the manufacturer.

The improved PCR-RFLP was carried out using the same PCR reagents as those in the conventional PCR-RFLP with forward (iRFLP-F) and reverse (iRFLP/PIRA-R) primers that were designed to reduce the number of digested DNA fragments compared to that in the conventional PCR-RFLP method (Table 1). After the first denaturation at 95ºC for 2 min, 40 cycles of amplification were carried out, at a denaturing temperature of 95ºC for 30 sec, an annealing temperature of 55ºC for 30 sec, and an extension temperature of 72ºC for 1 min. Extension during the last cycle was carried out for 6 min. The condition of restriction enzyme

digestion was the same as that in the conventional PCR-RFLP method.

PCR-PIRA method

The PCR-PIRA method can be used to detect a single nucleotide mutation by introducing an artificial restriction endonuclease site using primers containing mismatches (Haliassos et al., 1989). The PCR-PIRA assay in the present study was carried out with forward (PIRA-F) and reverse (iRFLP/PIRA-R) primers. The forward primer introduced the restriction site ($CT^{619}TAAG$) of restriction enzyme *Afl*II (New England Biolabs, Ipswich, MA.) on the mutant allele as a result of combination with the mutant genomic sequence (c.619T). The conditions of PCR and restriction enzyme digestion by *Afl*II (20 U) were the same as those in the improved PCR-RFLP method.

MS-PCR method

The MS-PCR method utilizes sequence-specific hybridization by 2 primers with widely different sizes and was originally developed for easier genotyping of human familial hypercholesteremia in a single PCR tube without a restriction endonuclease digestion process (Rust et al., 1993). In the present study, the MS-PCR assay was designed based on the general rules of MS-PCR (Rust et al., 1993), and the ratio of short to long allele-specific forward primers (MS-W-F and MS-M-F) was increased to 10:l on a molar basis (Table 1) in order to produce bands of similar intensity in the heterozygous carrier when visualized on gel

electrophoresis. The PCR assay was carried out using these 2 allele-specific forward primers and a reverse primer (MS-R) simultaneously in a single PCR tube in which the reaction condition was the same as that in the abovementioned PCR methods except for the annealing temperature (50°C).

Agarose gel and microchip electrophoreses

All undigested and digested PCR products except for the realtime PCR were subjected to electrophoresis in 3% (w/v) agarose gel (Certified Low Range Ultra Agarose; Bio-Rad Laboratories, Hercules, CA.) dissolved with Tris–EDTA buffer (10×TE Powder (pH 8.0), Wako Pure Chemical Industries Ltd., Osaka, Japan.). Molecular size markers (øX174/Hinf I digest, Nippon Gene, Tokyo, Japan.) were used in the agarose gel electrophoresis. The electrophoresed agarose gel was stained with ethidium bromide, and luminescence was induced by an ultraviolet transilluminator. Analysis of PCR products was also performed using a microchip electrophoresis system (MCE-202 MultiNA Microchip Electrophoresis System, Shimadzu Corp., Kyoto, Japan.) with a special reagent kit (DNA-500 Kit, Shimadzu Corp., Kyoto, Japan.) that included internal DNA size markers and DNA separation buffer. A fluorescent dye (SYBR Gold Nucleic Acid Gel Stain, Invitrogen Corp., Carlsbad, CA.) was added to the DNA separation buffer according to the manufacturer's protocol for the microchip electrophoresis system. The PCR products were diluted 10 times with deionized water before application to this system to diminish the adverse influence of PCR and endonuclease buffers on electrophoretic mobility. DNA ladder markers (25 bp DNA Ladder,

Invitrogen Corp., Carlsbad, CA.) from 25 to 450 base pairs (bp) were used as references for DNA sizing.

Real-time PCR method

Using a hole punch, a 1.2-mm in diameter disc was punched from the FTA cards described above (Whatman International Ltd, Piscataway, NJ.). The disc was placed into a separate 0.2-ml tube, lysed in the tube with 8 μl of lysis solution from a special DNA extraction kit (DNA Extract All Lysis Reagents Kit, Applied Biosystems, Foster City, CA.), and subsequently incubated at 95ºC for 3 min. Then, 8 μl of DNA stabilizing solution from the kit (DNA Extract All Lysis Reagents Kit, Applied Biosystems, Foster City, CA.) was added to the tube. This DNA-containing solution was transferred to a new tube and stored at –25ºC until analysis.

Amplifications were performed on a real-time PCR system (StepOne Real-Time PCR System, Applied Biosystems, Foster City, CA.) using a specific primer pair and TaqMan MGB probes bound with each fluorescent reporter dye (6-carboxyrhodamine or 6-carboxyfluorescein) at the 5'-end and a nonfluorescent quencher dye at the 3'-end (Table 1), which were synthesized by a commercial facility (TaqMan SNP Genotyping Assays, Applied Biosystems, Foster City, CA.). The real-time PCR amplifications were carried out in a final volume of 10 μl consisting of a master mix (2×) (TaqMan GTXpress Master Mix, Applied Biosystems, Foster City, CA.), a genotyping assay mix (80×) (TaqMan SNP Genotyping Assays, Applied Biosystems, Foster City, CA.) including specific primers and TaqMan MGB

probes, nuclease-free water, and 2 μl of the aforementioned DNA-containing solution as a DNA template. The holding stage before PCR was performed at 25ºC for 30 sec. The cycling conditions were 20 sec at 95ºC followed by 40 cycles of 3 sec at 95ºC and 20 sec at 60ºC. The holding stage after PCR was performed at 25ºC for 30 sec.

In addition, an allelic discrimination plot was constructed based on the 3 types of amplification plots. Those data were calculated using software (StepOne software (version 2.0), Applied Biosystems, Foster City, CA.) based on the results obtained using DNA samples from 46 Border Collies (19 noncarriers, 19 carriers, and 8 affected) in which the genotypes were determined by the other PCR tests developed in the present study.

Genotyping survey

The genotyping survey was carried out using DNA samples from saliva- or whole blood–spotted FTA cards of 469 Border Collies in Japan. The samples were randomly collected with owners' informed consent for scientific evaluation of their dogs' DNA. Sample collection was performed from 2006 to the present by the Japan Border Collie Health Network, a volunteer breeders' association for the healthy breeding of Border Collies. The genotypes were determined using one of the assays developed in the present study. Since the real-time PCR method was established, genotyping has been carried out using the real-time PCR assay only. The results obtained by the PCR-RFLP, PCR-PIRA, and MS-PCR assays were confirmed again by real-time PCR assay.

1.4. RESULTS

Conventional PCR-RFLP assay

In the conventional PCR-RFLP assay, a 290-bp DNA band was amplified in theory in all the genotypes (Fig. 1). Since the amplification product in the unaffected dog had 3 recognition sites of the restriction endonuclease *Mse*I, it was cleaved into 4 fragments (i.e., 129-, 101-, 38-, and 16-bp bands) after digestion with *Mse*I. In the affected dog, because there were 4 recognition sites of *Mse*I due to the mutation, the amplified band was cleaved into 5 fragments (i.e., 101-, 65-, 62-, 38-, and 16-bp bands), which appeared as 4 fragments due to the inseparability of 65- and 62-bp bands even on microchip electrophoresis (Fig. 1B). The amplification product in the carrier was cleaved into 6 fragments, which appeared as 5 fragments (i.e., 129-, 101-, 65-, 62-, 38-, and 16-bp bands). The 16-bp band was too small to be seen clearly under the screen of primer bands.

Improved PCR-RFLP assay

In the improved PCR-RFLP assay, a 322-bp DNA band was amplified in theory in all genotypes (Fig. 2). Because the amplification product in the unaffected dog had 1 recognition site of *Mse*I, it was cleaved into 2 fragments (i.e., 194- and 126-bp bands) after digestion. In the affected dog, because an additional restriction site due to the mutation was designed to be in the midst of the 126-bp fragment, the amplified band was cleaved into apparently 2

fragments (i.e., 194- and two 62-bp bands) after the digestion. The amplification product in the carrier was cleaved into apparently 3 fragments of 194-, 126-, and two 62-bp bands. Thus, the result of the improved PCR-RFLP assay provided simpler and clearer genotype discrimination compared to that in the conventional PCR-RFLP assay.

PCR-PIRA assay

In the PCR-PIRA assay, a 29-bp sequence corresponding to the forward primer was cut from the amplified 290-bp band after the digestion of *Afl*II, producing a 257-bp digested band in the mutant allele (Fig. 3). According to this theory, this assay produced a 290-bp undigested band in the unaffected dog, and 2 fragments (i.e., 290- and 257-bp bands) in the carrier. The 29-bp band was seen only on microchip electrophoresis in the affected dog because of its small band size and low density (Fig. 3A).

MS-PCR assay

As expected, in the MS-PCR assay, a 173-bp fragment was amplified with a wild allele-specific forward primer (49 mer) and a common reverse primer in the unaffected dog, and a 144-bp fragment with the mutant allele-specific forward primer (20 mer) in the affected dog (Fig. 4). In the carrier, these 2 bands of similar intensity were clearly seen on both agarose gel and microchip electrophoreses. In the gel image of microchip electrophoresis, a nonspecific band appeared in the midst of normal and mutant bands in the carrier (Fig. 4B),

but did not interfere with discrimination of the carrier genotype.

Real-time PCR assay

Real-time PCR assay with TaqMan MGB probes clearly determined all the genotypes of NCL in Border Collies without any nonspecific allelic amplification (Fig. 5). Forty cycles of amplification were sufficient for genotyping using either blood or saliva DNA samples on FTA cards. The total required time for 40-cycle amplification was within 40 min. In addition, an allelic discrimination plot was constructed based on the 3 genotypes of amplification plots obtained using DNA samples from 46 Border Collies including 19 noncarrier, 19 carrier, and 8 affected dogs (Fig. 6). Three genotypes of the 46 dogs were clearly determined by this allelic discrimination plot and the results were completely consistent with those of other PCR-based assays developed in the present study.

Allele frequency

In the genotyping survey carried out on 469 Border Collies in Japan, 33 dogs were heterozygous carriers, indicating that the carrier frequency was 7.0%. However, there were no affected dogs detected in this random survey, resulting in an allele frequency of 0.035.

1.5. DISCUSSION

In recessive inherited diseases such as lysosomal diseases, carriers that have one abnormal allele in the gene pair but have a normal clinical appearance are the most important genotype to identify because, although there are no physical clues to the presence of disease in these animals, the pathogenic mutation is transmitted to half of their progeny (Baker et al., 2001). The frequency of carriers in a population substantially exceeds the incidence of affected individuals and thus the carrier state makes recessive disease the most dangerous of all patterns of inheritance in purebred animals (Yamato et al., 2009). Therefore, to prevent and eradicate fatal hereditary diseases, the determination of genotypes through systematic monitoring surveys and continuous removal of carriers from breeding colonies might be the most important and efficient measures. In the present study, rapid and reliable assays were developed to determine the genotypes of NCL in Border Collies and the current allele frequency in Japan was surveyed to examine the necessity of disease control.

The conventional genetic assay reported previously (Melville et al., 2005) was not a reliable method for easily discriminating the genotypes because of the complex and unclear DNA fragmentation (Fig. 1). In the improved PCR-RFLP assay, the mutant homozygote could be easily discriminated from the noncarrier and carrier genotypes because a 126-bp fragment was absent in affected dogs only, but, this assay showed a lower reliability in discriminating between noncarrier and carrier genotypes because the judgment depends on the absence or presence of a slightly weak 62-bp band (Fig. 2). In the PCRPIRA assay, it was relatively easy to discriminate between carrier and other genotypes because the carrier genotype clearly had double DNA fragments of 290- and 257-bp (Fig. 3). However, the difference between these 2

band sizes was only 33-bp, making it slightly difficult to discriminate between normal and affected genotypes especially on agarose gel electrophoresis (Fig. 3A), but the judgment was easier using microchip electrophoresis (Fig. 3B). The MS-PCR assay clearly demonstrated the genotypes in a single tube without any restriction endonuclease digestion unlike the PCR-RFLP and PCR-PIRA assays (Fig. 4). Therefore, the MS-PCR assay was more rapid, simpler, and less expensive than PCR-RFLP and PCR-PIRA assays.

Microchip electrophoresis has recently attracted considerable attention in DNA analysis because of its high efficiency, high throughput, timesaving ability, easy operation, and low consumption of samples and reagents (Chang et al., 2010b; Rahman et al., 2011; Zhang et al., 2003). The use of microchip electrophoresis can markedly shorten the time for analysis of DNA fragment patterns, which is approximately 3 min per sample. In the present study, the discrimination of genotypes was more rapid and clearer using microchip electrophoresis rather than agarose gel electrophoresis because of its high resolution and detection sensitivity (Figs. 2B, 3B, and 4B). On MS-PCR assay, a nonspecific band appeared in the midst of normal and mutant bands in the carrier (Fig. 4B). This was regarded as heteroduplex of PCR products for wild-type and mutant alleles. This band might be detected by the high sensitivity of the microchip electrophoresis, but it is less of an obstacle to the judgment of carrier genotype.

Recently, rapid real-time quantitative PCR approaches have been developed to detect mutations in genes causing hereditary diseases in human beings (Joncourt et al., 2004; Vrettou et al., 2004) and animals (Chang et al., 2010a, 2010b). Real-time PCR is generally considered more sensitive, rapid, and less time-consuming than PCR in combination with electrophoresis

analysis (Chang et al., 2010a, 2010b; Katz et al., 2011; Vrettou et al., 2004). In particular, real-time PCR assay in combination with FTA cards for sampling can markedly shorten the time required for genotyping and simplify the procedure (Chang et al., 2010a). In the present study, therefore, the application of real-time PCR with TaqMan MGB probes was investigated to establish a rapid and reliable genotyping technique for NCL in Border Collies. As a result, this assay clearly determined all genotypes (Fig. 5). In addition, an allelic discrimination plot analysis yielded multiple results simultaneously (Fig. 6), which can be particularly useful for large-scale epidemiological survey and preventive screening as well as diagnosis of affected dogs.

In domestic animals, there are few reports of the carrier frequency in fatal inherited diseases. In a preliminary genotyping survey carried out in Shiba dogs on a single mutation of GM1 gangliosidosis, one of the lysosomal storage diseases, the carrier frequency in Japan was reported to be 2.9% (Yamato et al., 2008). In mixed breed cats in Japan, it is reported that the allele frequency for a single mutation of GM2 gangliosidosis variant 0 is less than 0.1% (Rahman et al., 2011). In the present study, the carrier frequency of the canine *CLN5* single mutation (c.619C>T) was 7.0% in the population of Border Collies in Japan. Although there is no objective criterion to evaluate the degree of frequency, the carrier frequency of NCL in Border Collies in Japan may be very high compared with those in other human and animal lysosomal diseases. This high carrier frequency suggests the presence of a founder effect in the population of Border Collies in Japan.

In conclusion, the present study demonstrates that 4 types of genotyping assays including improved PCR-RFLP, PCR-PIRA, MS-PCR, and real-time PCR are useful for diagnosis and

screening of NCL in Border Collies. In particular, real-time PCR assay with TaqMan MGB probes is suitable for largescale preventive screening. Using these assays, measures to prevent the continuing spread of this fatal inherited disease should be undertaken in the population of Border Collies.

Table 1. Characteristics of primers and TaqMan probes used in the present study.

Name*	Assay*	Primer/probe	Sequence† 5'→3' (mer)	Location on ORF‡	Tm (°C)	Concentration (nM)
cRFLP-F	Conventional PCR-RFLP	Forward	TTTGCTTTGGTGTTCACATAGG (22)	Intron 3	54.8	625
cRFLP-R	Conventional PCR-RFLP	Reverse	CCCAAGTAGGTAGGTTCTCCA (21)	765-785	58.5	625
iRFLP-F	Improved PCR-RFLP	Forward	AAATGGCAAAGTGGGTAAAGCGGG (24)	557-580	60.5	625
PIRA-F	PCR-PIRA	Forward	ACAGGAATTTATTACGAGACGTGGACTG**C**T (30)	589-618	61.9	625
iRFLP/PIRA-R	Improved PCR-RFLP & PCR-PIRA	Reverse	GTTGATAAATGTGGTTTGAAGGGG (24)	855-878	57.1	625
MS-W-F	MS-PCR	Forward for wild allele	GTAAAGCGGGACAATGAAACAGGAATTTA**CC**ACGAGACGTGGACTG**C**TC (49)	571-619	70.7	62.5
MS-M-F	MS-PCR	Forward for mutant allele	TTACGAGACGTGGACTGT**C**T (20)	600-619	56.3	625
MS-R	MS-PCR	Reverse	GTTGGTTTCTATCTTCTTGAACTC (24)	718-741	55.3	625
RT-F	Real-time PCR	Forward	GCGGGACAATGAAACAGGAATTTAT (25)	576-600	56.1	450
RT-R	Real-time PCR	Reverse	TGTCTCAGCCCCCTTTGTTG (20)	629-648	57.6	450
Probe-VIC-W	Real-time PCR	Probe for wild allele	CTGGCTTGAACAGTCC (16)	611-626	-	100
Probe-FAM-M	Real-time PCR	Probe for mutant allele	CTGGCTTAAACAGTCC (16)	611-626	-	100

* PCR = polymerase chain reaction; RFLP = restriction fragment length polymorphism; PIRA = primer-induced restriction assay; MS = mutagenically separated; RT = real-time; F = forward; R = reverse; W = wild-type; M = mutant-type; VIC = 6-carboxyrhodamine; FAM = 6-carboxyfluorescein. † Underlined letters in the sequence of primers are mismatched to that of canine *CLN5* gene. ‡ ORF: open reading frame, which is the 1053-base pair sequence (GenBank accession No. NM001011556).

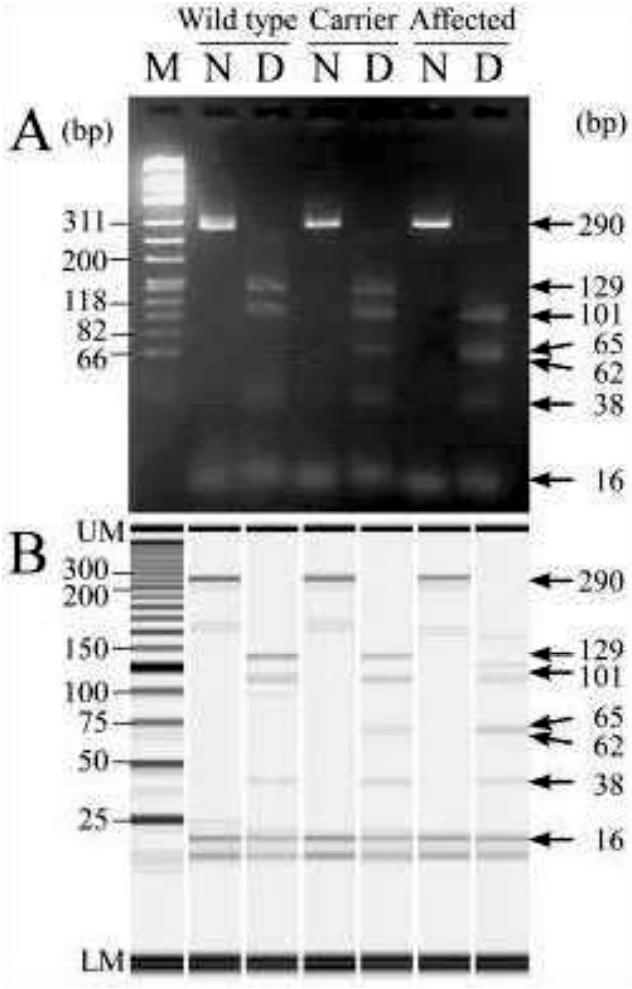

Figure 1. Neuronal ceroid lipofuscinosis genotyping of wild-type, heterozygous carrier and affected dogs by the conventional PCR-restriction fragment length polymorphism assay using agarose gel electrophoresis (A) and microchip electrophoresis (B). The amplified DNA without digestion (N) and DNA digested with restriction endonuclease *Mse*I (D) were analyzed simultaneously by both methods of electrophoresis. Lane M shows molecular size markers and base pairs (bp). UM and LM show upper and lower markers, respectively, contained readily in the DNA separation buffer of the commercial kit.

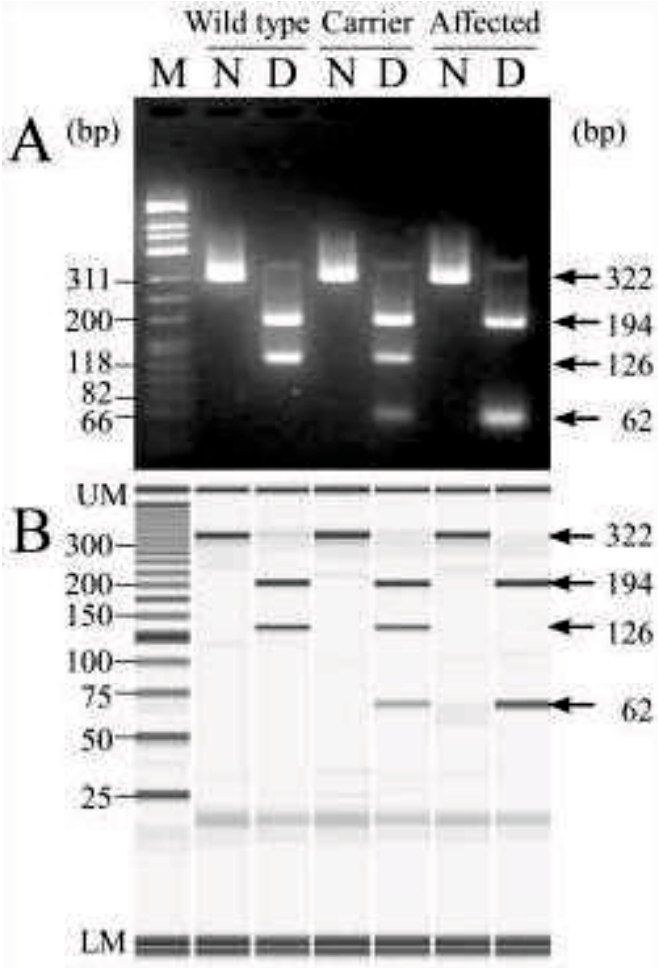

Figure 2. Neuronal ceroid lipofuscinosis genotyping of wild-type, heterozygous carrier and affected dogs by the improved PCR-restriction fragment length polymorphism assay using agarose gel electrophoresis (A) and microchip electrophoresis (B). The amplified DNA without digestion (N) and DNA digested with restriction endonuclease *Mse*I (D) were analyzed simultaneously by both methods of electrophoresis. Lane M shows molecular size markers and base pairs (bp). UM and LM show upper and lower markers, respectively, contained readily in the DNA separation buffer of the commercial kit.

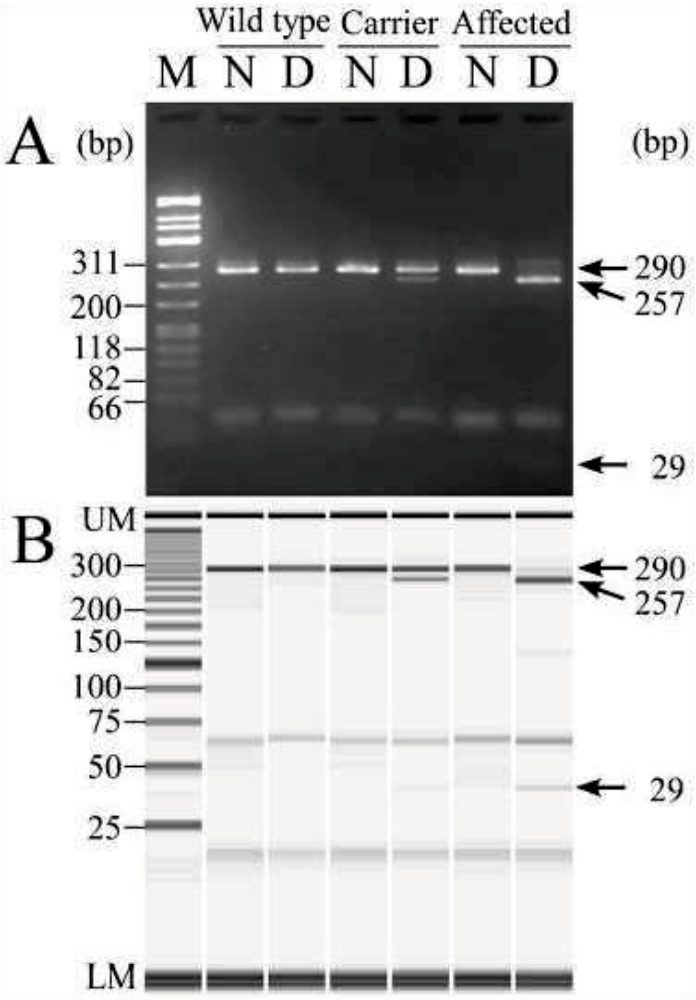

Figure 3. Neuronal ceroid lipofuscinosis genotyping of wildtype, heterozygous carrier and affected dogs by the PCR-primer induced restriction analysis assay using agarose gel electrophoresis (A) and microchip electrophoresis (B). The amplified DNA without digestion (N) and DNA digested with restriction endonuclease *Afl*II (D) were analyzed simultaneously by both methods of electrophoresis. Lane M shows molecular size markers and base pairs (bp). UM and LM show upper and lower markers, respectively, contained readily in the DNA separation buffer of the commercial kit.

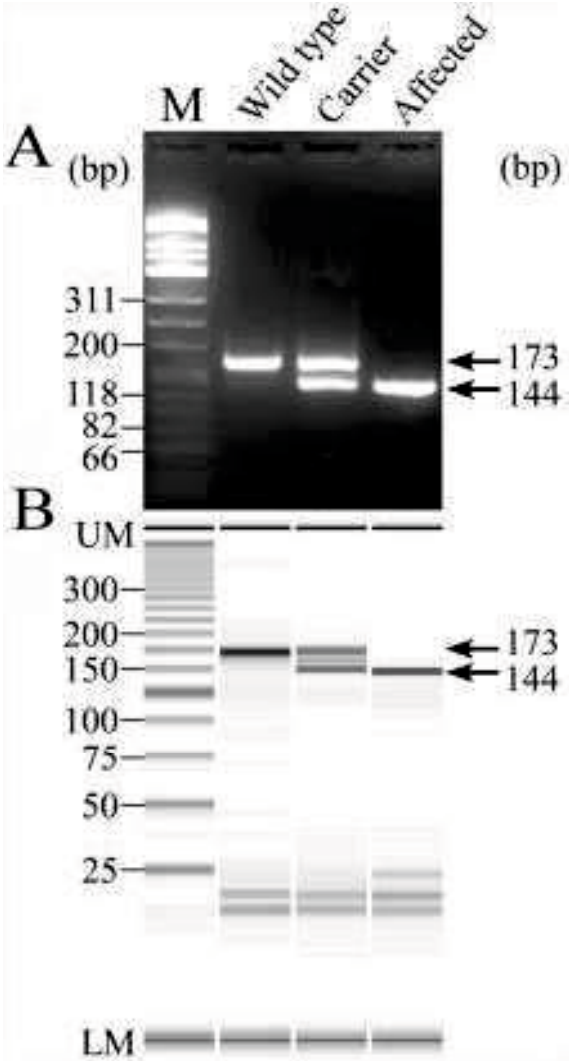

Figure 4. Neuronal ceroid lipofuscinosis genotyping of wildtype, heterozygous carrier and affected dogs by the mutagenically separated PCR assay using agarose gel electrophoresis (A) and microchip electrophoresis (B). Lane M shows molecular size markers and base pairs (bp). UM and LM show upper and lower markers, respectively, contained readily in the DNA separation buffer of the commercial kit.

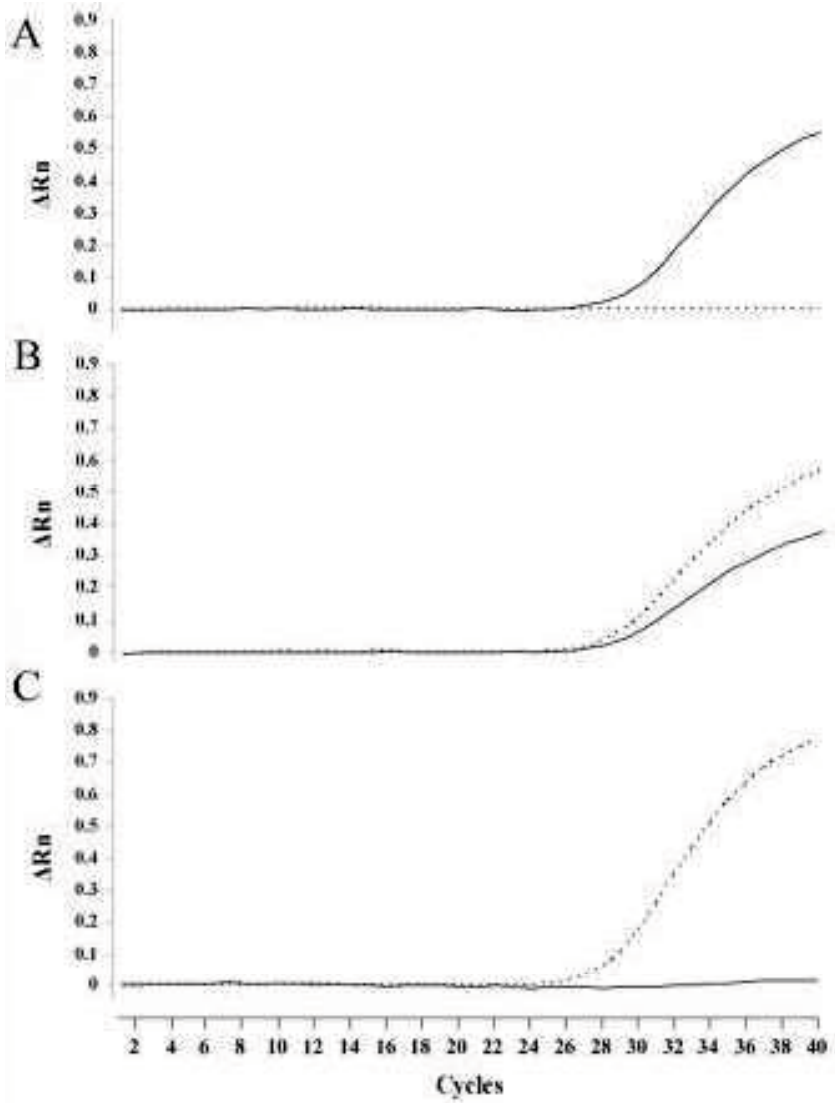

Figure 5. Real-time PCR amplification plots of wild-type and mutant alleles in canine neuronal ceroid lipofuscinosis. Amplification was plotted as fluorescence intensity (ΔRn value) against cycle number. The ΔRn value is the reporter dye signal normalized to internal reference dye and corrected for the baseline signal established in the first few cycles of reaction. Each of 3 amplification plots showed the normal (A), heterozygous carrier (B), and homozygous affected dogs (C). Solid and dotted lines indicate wild-type and mutant alleles, respectively.

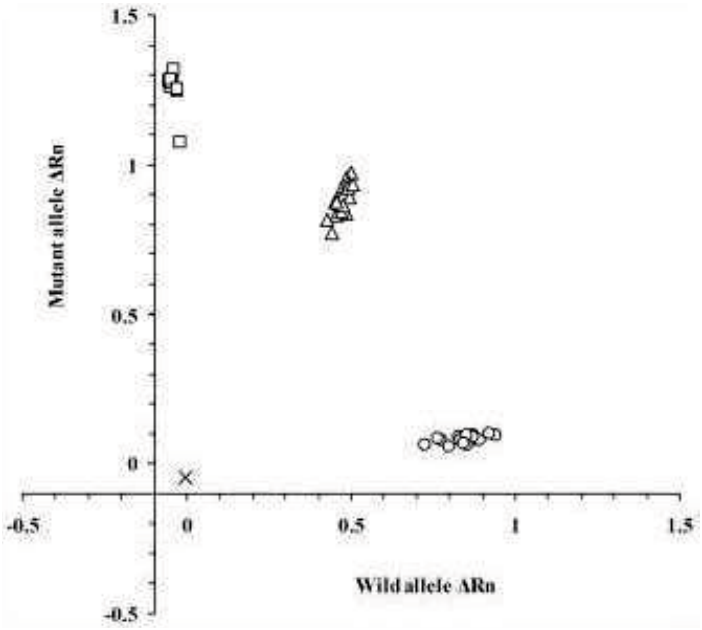

Figure 6. Allelic discrimination plot of end-point fluorescence real-time PCR data showing the 3 genotypes of canine neuronal ceroid lipofuscinosis. Allelic discrimination plot was depicted using representative 46 DNA samples in Border Collie dogs that had already been genotyped by other assays developed in the present study. The plot is expressed as fluorescence intensities (ΔRn value) for each allele at the X- and Y-axes. The ΔRn value in this figure is the end-point reporter dye signal normalized to internal reference dye and corrected for the baseline signal established in the first few cycles of reaction. ×, no template control; ○, normal genotype (19 samples); Δ, carrier genotype (19 samples); □, affected genotype (8 samples).

CHAPTER 2

Clinical and molecular epidemiological study of neuronal ceroid lipofuscinosis in Border Collies

The above-titled work originally appeared in "*The ScientificWorld Journal* (Mizukami et al., 2012)" as: ***Neuronal Ceroid Lipofuscinosis in Border Collie Dogs in Japan: Clinical andMolecular Epidemiological Study (2000–2011)*** authored by:

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2.1. ABSTRACT

Neuronal ceroid lipofuscinosis (NCL) is an inherited, neurodegenerative lysosomal disease that causes premature death. The present study describes the clinical and molecular epidemiologic findings of NCL in Border Collies in Japan for 12 years, between 2000 and 2013. The number of affected dogs was surveyed, and their clinical characteristics were analyzed. In 4 kennels with affected dogs, the dogs were genotyped. The genetic relationships of all affected dogs and carriers identified were analyzed. The survey revealed 27 affected dogs, but there was a decreasing trend at the end of the study period. The clinical characteristics of these affected dogs were updated in detail. The genotyping survey demonstrated a high mutant allele frequency in examined kennels (34.8%). The pedigree analysis demonstrated that all affected dogs and carriers in Japan are related to some presumptive carriers imported from Oceania and having a common ancestor. The current high prevalence in Japan might be due to an overuse of these carriers by breeders without any knowledge of the disease. For NCL control and prevention, it is necessary to examine all breeding dogs, especially in kennels with a high prevalence. Such endeavors will reduce NCL prevalence and may already be contributing to the recent decreasing trend in Japan.

2.2. INTRODUCTION

Neuronal ceroid lipofuscinosis (NCL) is a rare group of inherited, neurodegenerative lysosomal storage diseases characterized histopathologically by the abnormal accumulation of ceroid- or lipofuscin-like autofluorescent lipopigments in neurons, retinal cells, and other visceral cells throughout the body (Jolly et al., 1994, 1995; Hofmann and Petronen, 2001; Jalanko and Braulke, 2009). NCL shares certain clinical features in both human beings and animals, including behavioral abnormalities, such as personality changes and aggressiveness, mental retardation and/or dementia; motor disturbances, such as ataxia and incoordination; visual problems leading to central and/or retinal blindness; premature death, but these differ in degree based on the causative gene, of which there are currently at least 8, all recessively inherited (Jalanko and Braulke, 2009). NCL has been described in several domestic species and occurs most commonly in dogs (Jolly et al., 1994, 1995; Mizukami et al., 2011).

NCL in Border Collies was first identified in Australia in the 1980s (Taylor and Farrow, 1988, 1992; Studdert and Mitten, 1991), and a sporadic case with the disease was also reported in the USA in the 1990s (Franks et al., 1999). A diagnosis of the first case in Japan was made in a Border Collie that was born in 2000 (Koie et al., 1994). The pathogenic mutation was reported in 2005 to be a nonsense mutation (c.619C>T) in exon 4 in the canine *CLN5* gene (Melville et al., 2005), which enabled a DNA-based genotyping of affected dogs and carriers. Recently, several types of rapid genotyping assays for this mutation were developed, and the carrier frequency (8.1%) in Japan was determined by a genotyping survey using these assays, suggesting the mutant allele frequency of NCL in Border Collies is high enough in Japan that measures to control and prevent the disease would be warranted

(Mizukami et al., 2011).

The present study describes the clinical and molecular epidemiologic findings of NCL in Border Collies in Japan for 12 years, between 2000 and 2011. This study also discusses the control and prevention of the disease based on the results of these analyses.

2.3. MATERIALS AND METHODS

Dogs Affected with NCL

The number of affected dogs was surveyed based on the records in our laboratory, which have been exclusively supporting the diagnosis of the disease in Japan. Among 27 affected dogs identified in the present study, NCL was diagnosed definitively in 25 dogs by a genetic test (Mizukami et al., 2011) using their specimens containing DNA, but in the remaining 2 dogs that died without any specimens before the genetic test got available, NCL was strongly suspected based on their typical clinical history and blood relationship with molecularly defined affected littermates and/or carrier parents. The clinical characteristics were analyzed and summarized using information about all of the affected dogs, which was gleaned from interviews and questionnaires of their owners and veterinarians. Some of the affected dogs were examined using the following prediagnostic tests: magnetic resonance image (MRI) scan in 7 dogs, including a previously reported dog (Koie et al., 1994); computed tomography (CT) scan in 2 dogs; ophthalmologic examination in 3 dogs. The findings of these examinations were also analyzed and summarized.

Genotyping Survey

Border Collies belonging to 4 special kennels (A, B, C, and D), which generated one or more affected dogs, were surveyed between 2008 and 2010 using a genetic test with the

breeders' cooperation. The number of dogs examined was 82, including 23, 20, 29, and 10 in kennels A, B, C, and D, respectively. Whole blood or saliva specimens were collected from these dogs using the Flinders Technology Associates filter paper (FTA card, Whatman International). Genotyping was performed as reported previously (Mizukami et al., 2011).

Pedigree Analysis

Pedigree analysis was performed to elucidate the genetic relationships of affected and carrier dogs identified in Japan and to deduce the pathway of transmission and distribution of the mutant allele. The genetic relationships among affected and carrier dogs found in the present study were analyzed using the pedigree papers issued by the Japan Kennel Club (http://www.jkc.or.jp/) and the Kennel Club of Japan (http://www.kcj.gr.jp/). The pedigree information of carrier dogs identified in the previous random survey in Japan (Mizukami et al., 2011) was analyzed and added to the results of the present study. The pedigree information of the dogs was used under the informed consent of their owners. The ancestry in Oceania was traced using pedigree information of carrier dogs provided by the New South Wales Border Collie Club (http://www.mybcsite.com/bccnswwebfiles/bccnswframe.htm), which was also disclosed via the website of the Japan Border Collie Health Network (http://www.jbchn.net/cl carrier.htm), a volunteer breeders' association for the healthy breeding of Border Collies. Pedigree information published in the Border Collie Database (http://db.kennel.dk/) was used to supplement the information about the ancestor dogs in Oceania.

All animals were cared for and were used in the experiments in accordance with the

guidelines for proper conduct of animal experiments issued by the Science Council of Japan (http://www.scj.go.jp/en/animal/index.html). All experimental procedures using animals and their specimens were performed in accordance with the guidelines regulating animal use at Kagoshima University.

2.4. RESULTS

The Number and Clinical Characteristics of Border Collies with NCL

The survey revealed that there were 28 affected dogs (14 males and 14 females) in the 14 years from 2000 to 2013 in Japan (Table 2). The first case, born in 2000, was diagnosed with NCL histopathologically (Koie et al., 1994), which was confirmed molecularly using the stored liver specimen. Since this case, several affected dogs from a single or a few litters were diagnosed nearly every year up until recently in Japan. However, currently there seems to be a decreasing trend based on the observation that only 2 affected dog was identified since 2009.

The clinical features of the affected dogs are summarized and listed in Table 3. They were divided into 3 stages, that is, early (15–20 months of age), middle (19–23 months of age), and late-to-terminal stages (22–32 months of age). The overlaps in age between adjacent stages were due to individual variability in the onset and progression rate of the disease. The features at the early stage were mainly behavioral problems that most owners of the dogs did not regard as pathological changes. The features at the middle stage included serious behavioral abnormalities, visual impairments, and slight motor disorders, which prompted the owners to consult veterinarians. The features at the lateto-terminal stage included serious motor, visual, psychointellectual, and vital dysfunctions due to a wide spectrum of brain dysfunctions. The mean life span of the 25 affected dogs examined was 26.3 months of age. Of these, 18 died naturally at a mean age of 26.8 months, ranging from 23 to 32 months. The other 7 dogs were euthanized at the terminal stage an average of 25.1 months. Although there were few specific

changes before 15 months of age, some of the owners had an impression that their dogs had unusual characteristics and behaviors and low learning ability in the puppy and juvenile years. There was no abnormal change on general clinico-pathological examinations. No abnormal vacuoles in the cytoplasm of leukocytes were observed in blood smear examinations.

MRI, CT, and Ophthalmologic Examinations

MRI, CT, and ophthalmologic examinations were performed in affected dogs in the middle-to-late stage (at approximately 2 years of age). The common findings of MR and CT images were ventricular enlargement and well-demarcated cerebral sulci (Fig. 7). Dilation of cerebellar fissures was also observed at a sagittal section of the MRI scan (Fig. 7(c)). No other organic lesion was detected in MRI and CT scans. In the affected dogs examined ophthalmologically, slight narrowing of blood vessels in the retina was commonly observed in ophthalmoscopy (Fig. 8(a)), but this ophthalmoscopic finding was evaluated within the normal range by veterinary ophthalmologists. An examination using a slit lamp detected no abnormal finding, such as clouding of the cornea and lens (Fig. 8(b)).

Genotyping Survey

The results of the genotyping survey in the 4 specific kennels are shown in Table 4. The frequencies of carriers (32.9%), affected dogs (18.3%), and the mutant allele (34.8%) were markedly high in the 4 kennels.

Pedigree Analysis

The pedigree analysis was performed mainly using the pedigree information of the 27 affected dogs and 58 carriers identified so far in Japan (Fig. 9). These dogs were related to at least 13 possible carriers imported from Oceania to Japan in the middle 1990s, which ultimately revealed an ancestry from a male dog born in 1944 in Australia through 9 carriers reported by the New South Wales Border Collie Club. As a result, this analysis revealed that all dogs carrying the mutant allele in Japan share a blood relationship and a single common ancestor in Australia.

The pedigree analysis also revealed that breeding was repeated using a small number of carriers and their offspring in the 4 kennels surveyed in the present study. The breeders of these kennels were not aware of NCL in Border Collies before the genotyping survey. As a result, these kennels had markedly high frequencies of the mutant allele.

2.5. DISCUSSION

The present study revealed 28 Border Collies (14 males and 14 females) with NCL in Japan during 2000–2011 (Table 2), although there might have been additional affected dogs elsewhere that had no opportunity to be diagnosed. Since the first affected dog, born in 2000, was diagnosed in 2002 (Koie et al., 1994), several affected dogs from a single or a few litters have been diagnosed nearly every year up until recently, but currently there seems to be a decline in the frequency of NCL. The decreasing trend may be due to the publication of the pathogenic mutation in 2005 (Jolly and Palmer, 1995), the subsequent development of a variety of genetic testing methods (Mizukami et al., 2011), and genotyping surveys in a random population of breeding Border Collies (Mizukami et al., 2011) and in a few specific kennels that generated affected dog(s) (present study). These events may have contributed to the prevention and control of the disease through the education of related breeders and fanciers of Border Collies.

Based on information from the owners and veterinarians of the affected dogs, their characteristic clinical features were determined (Table 3), although there were individual differences in the onset and progression rate of the disease and the symptoms, as reported previously (Taylor and Farrow, 1988).Mild clinical signs, such as behavioral problems, begin at 15 months of age at the earliest, but the onset is usually a few months later (approximately 18 months of age) at the early stage. The earliest age of onset in the present study is consistent with that in a previous report (Taylor and Farrow, 1992). Clinical signs observed at the early stage (15–20 months of age) aremainlymild behavioral abnormalities that the owners, without any knowledge of NCL, hardly regard as pathological. Visual disorders become unambiguous

and behavioral abnormalities increase in severity at the middle stage (19–23 months of age). Serious clinical signs, such as convulsive seizure and motor disorders, appear at the late-to-terminal stage (older than 22 months of age). The mean life span of affected dogs was 26.3 months of age in the present study. The life span was 26.8 months of age in the dogs that died naturally, ranging from 23 to 32 months of age. This is longer than the 23.1-month life span of affected Border Collies in a previous report in Australia (Studdert and Mitten, 1991). The difference could be attributed to the fact that many Japanese pet owners feel that euthanasia is inhumane and do not want to kill animals before they die naturally.

The characteristic clinical features determined in the present study are similar to those in previous reports (Taylor and Farrow, 1988; Studdert and Mitten, 1991). However, a previously reported clinical sign, mania (Studdert and Mitten, 1991), was not observed in the present study. In addition, some affected dogs had unusual characteristics and behaviors and low learning ability in the puppy and juvenile years, which was not reported previously. These clinical characteristics, updated in detail, will be of further help for the diagnosis of NCL in Border Collies.

In the MRI examination, ventricular enlargement and dilated cerebral and cerebellar sulci were common findings in affected dogs (Fig. 7). The CT examination also demonstrated ventricular enlargement. These characteristic findings suggest atrophy of the forebrain, which was similarly observed in previous MRI (Koie et al., 1994) and CT examinations (Franks et al., 1999). The changes suggesting brain atrophy developed as early as the middle stage, when affected dogs were usually referred to animal hospitals. Similar changes have been observed in other types of NCL (Kuwamura et al., 2003; Nakamoto et al., 2011) and other lysosomal

storage diseases, such as GM1 gangliosidosis (Hasegawa et al., 2012) and GM2 gangliosidosis (Matsuki et al., 2005; Hasegawa et al., 2007; Tamura et al., 2010) in dogs and cats. The brain atrophy usually develops as a specific and severe change even at the middle stage in NCL but as a secondary and mild change in the late-to-terminal stage in other lysosomal diseases. Therefore, the atrophic changes would be useful as an adjunct to the diagnosis of NCL. No other organic lesion was detected in MRI scans in Border Collies with NCL, although hyperintensity in T2-weighted images of the cerebral white matter is observed in GM1 and GM2 gangliosidoses in dogs and cats (Hasegawa et al., 2012; Hasegawa et al., 2007; Tamura et al., 2010; Kaye et al., 1992; Kroll and Pagel, 1995). Meningeal thickening, reported in Chihuahuas with NCL (Nakamoto et al., 2011), was not observed in Border Collies with NCL.

Visual impairments appeared to various degrees in all affected dogs, especially at the middle stage (Table 3). Previous reports have not described any abnormal changes on ophthalmoscopic examination (Studdert and Mitten, 1991; Koie et al., 1994). In the present study, the affected dogs examined had slight narrowing of blood vessels in the retina (Fig. 8(a)), but this ophthalmoscopic finding was considered within the normal range by veterinary ophthalmologists (Fig. 8). In humans with NCL, there is a pronounced loss of photoreceptors in the end stage of the disease, but in NCL in English Setters only minimal structural damage is observed in the retina (Koppang, 1988). In Border Collies with NCL, inclusions with variable ultrastructure are common in all cells of the retina, but the pigment accumulation does not damage the retinal architecture (Taylor and Farrow, 1992). The retinal lesions in the Border Collies are similar to those in the English Setters but are much less severe than in

juvenile NCL in humans. The narrowing of retinal blood vessels observed in affected Border Collies may be an indication of mild retinal degeneration, but this feature cannot be used as a valuable diagnostic for NCL.

Based on data of the pedigree analysis, affected dogs and carriers identified in Japan share a blood relationship and a single common ancestor born in 1944 in Australia via some presumptive carriers in Oceania (Fig. 9). Although it is impossible to demonstrate whether this ancestor was a founder of NCL in Border Collies, there is a possibility that the mutant allele was transmitted from Oceania to Japan in this way mainly in the middle of the 1990s, when the causative mutation had not been identified yet. The recent increase in prevalence of the disease in Japan is likely due to an overuse of imported carriers and their offspring as breeding dogs without any knowledge of the disease. Hereafter, dogs that will be bred should be genotyped beforehand.

The pedigree analysis also suggests that breeding has been repeated using a small number of carriers and their offspring in the 4 kennels that generated affected dogs (Fig. 9). There was a trend toward inbreeding in these kennels. In addition, the breeders of the kennels were not aware of the disease until they were informed of the generation of affected dogs in their kennels. These issues may have caused the high frequencies of carriers (32.9%) and the mutant allele (34.8%) in the 4 kennels (Table 3), compared to the carrier (8.1%) and mutant allele frequencies (4.1%) in the random population of Border Collies (Mizukami et al., 2011). Fifteen (55.6%) of the 27 affected dogs identified so far in Japan were generated in these 4 kennels. It is thought that affected dogs are generated in a small proportion of kennels, which have a high frequency of themutant allele. Therefore, it is important for prevention of the

disease to rapidly genotype all breeding dogs in kennels that have had an opportunity to generate affected dogs. This type of examination helps the kennels not only stop generating additional affected dogs but also stop spreading carriers to other kennels. In addition, a genotyping test using specimens from a random population of breeding Border Collies should be continued (Mizukami et al., 2011) to detect sporadic carriers and prevent them from being used as breeding dogs. These approaches would gradually decrease the number of dogs carrying the mutant allele. These active and continuous preventive measures may be necessary to eliminate NCL in Border Collies.

Table 2. Number of Border Collies with neuronal ceroid lipofuscinosis and the number of litters that included affected dogs during the 12 years between 2000 and 2013 in Japan.

Year of birth	Number of affected dogs (male, female)	Number of litters
2000	2 (2, 0)	1
2001	6 (3, 3)	3
2002	0 (0, 0)	0
2003	4 (3, 1)	3
2004	4 (1, 3)	2
2005	2 (1, 1)	2
2006	1 (0, 1)	1
2007	2 (1, 1)	2
2008	5 (2, 3)	4
2009	1 (0, 1)	1
2010	1 (1, 0)	1
2011	0 (0, 0)	0
2012–present*	0 (0, 0)	0
Total	28 (14, 14)	20

* At present (May 2013), dogs born since January 2012 are younger than 17 months of age, and therefore, some of the affected dogs have not reached the onset age (15–20 months of age) of neuronal ceroid lipofuscinosis in Border Collies.

Table 3. Summary of clinical features in Border Collies with neuronal ceroid lipofuscinosis.*

Stage (months of age)	Clinical signs
Early (15–20)	Altered characteristics; disregard for owner's commands; loss of interest in play and other dogs; morbid fear of noise, humans, and unspecified things; hallucination; disorientation; biting; averse to going up and down stairs (especially down).
Middle (19–23)	Fly-biting behavior; continuous shaking of the head; sudden halts during walks; uncooperativeness with other dogs; head tilt; chomping without food; tooth grinding; leg jerking; visual impairments (afraid of darkness, unawareness of things such as food, and frequently hitting obstacles); aggressiveness; excitation; staggering; falling; toileting accidents; myoclonus; myoclonic seizure.
Late to terminal (22–32)	Wandering; hair-pulling disorder; circadian rhythm disorder; acoustic and cutaneous hyperesthesia; cognitive and emotional impairments; blindness; dysmetria; gait deficiency; convulsive seizure; face and mouth tic; chewing difficulty; lethargy; stupor; death (mean age 26.8 months, ranging from 23 to 32 months)†.

* Data summarized from information of 28 affected dogs.

† Data from 18 affected dogs that died naturally without euthanasia.

Table 4. Results of the genotyping survey carried out in 4 kennels that generated affected dogs.*

Kennel	Number			Frequency (%)		
	Dogs examined	Carriers	Affected dogs	Carriers	Affected dogs	Mutant allele
A	23	9	8	39.1	34.8	54.3
B	20	11	2	55.0	10.0	37.5
C	29	6	2	20.7	6.7	17.2
D	10	1	3	10.0	30.0	35.0
Total	82	27	15	32.9	18.3	34.8

* These genotyping surveys were carried out between 2008 and 2010.

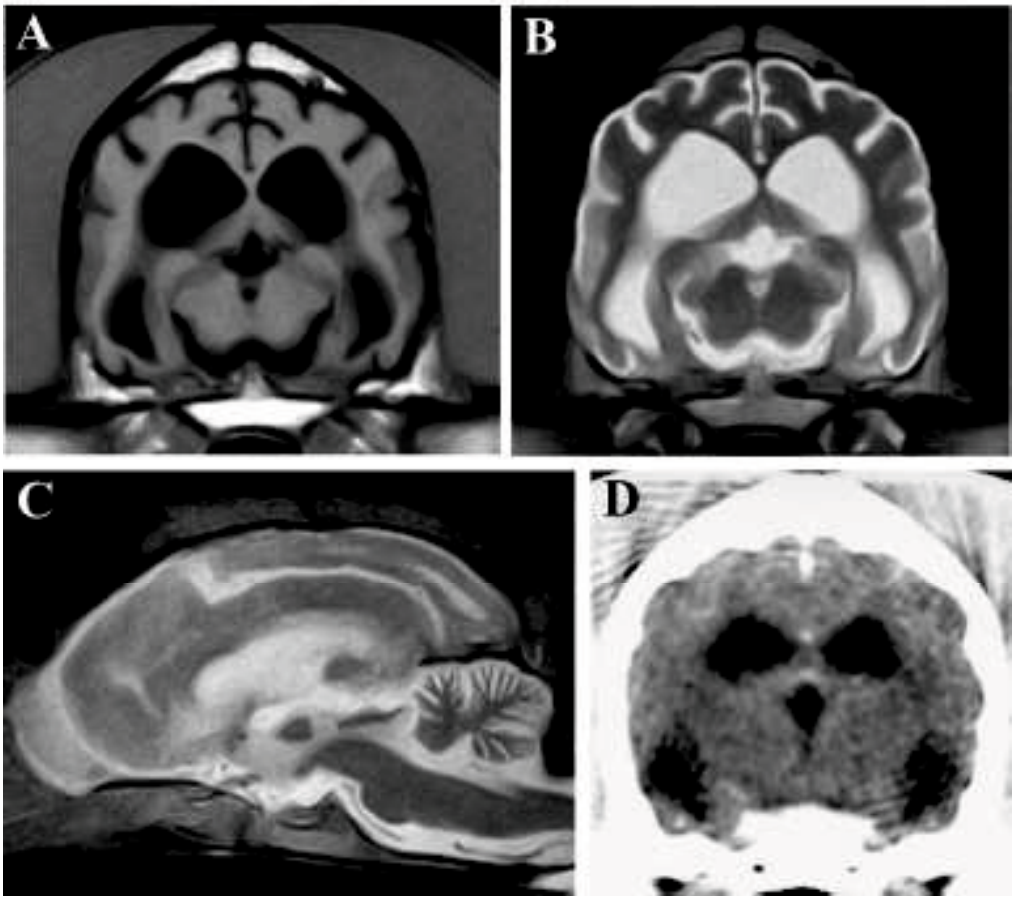

Figure 7. Representative magnetic resonance (MR) and computed tomography (CT) images of the brain of Border Collies with neuronal ceroid lipofuscinosis. MR images were obtained using a 0.3-tesla system (AIRIS2-comfort, HitachiMedical Corporation) in a 24-month-old dog under general anesthesia. CT images were obtained using a multislice CT system (ECLOS, Hitachi Medical Corporation) in another 24-month-old dog under general anesthesia. (A) MR T1-weighted image (TR/TE = 500/20 ms) of a transverse section at the level of the thalamus, (B) MR T2-weighted image (TR/TE = 4,000/120 ms) of a transverse section at the level of the thalamus, (C) MR T2-weighted image of a sagittal section, and (D) CT image of a transverse section at the level of the thalamus. These images show enlarged ventricles and dilated cerebral and cerebellar sulci, suggesting forebrain atrophy.

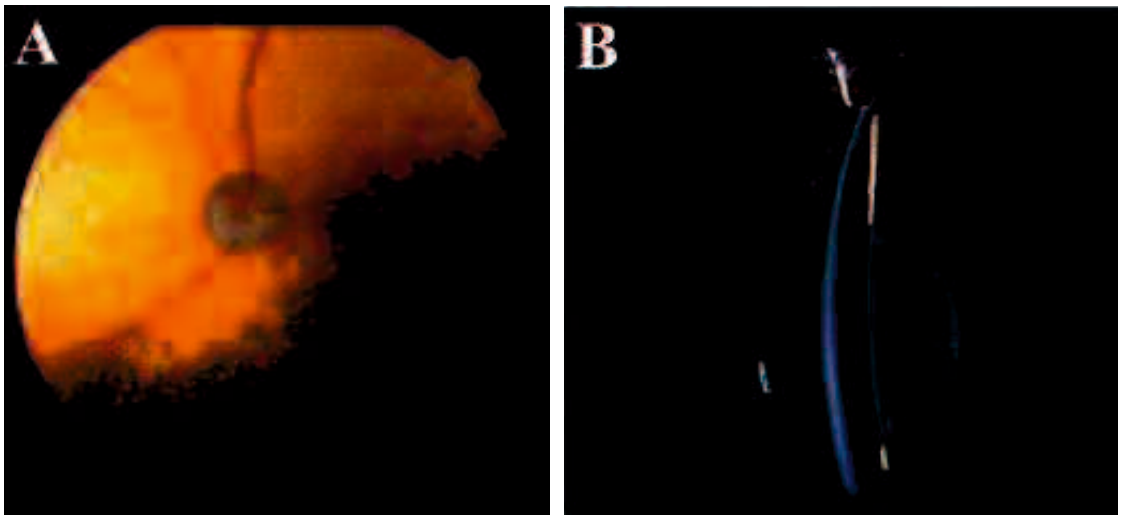

Figure 8. Photographs of ophthalmoscopic and slit-lamp examinations of the right eye in a 21-month-old Border Collie with neuronal ceroid lipofuscinosis. (a) Slight narrowing of blood vessels in the retina is observed, but (b) there is no abnormal finding in the slit-lamp examination.

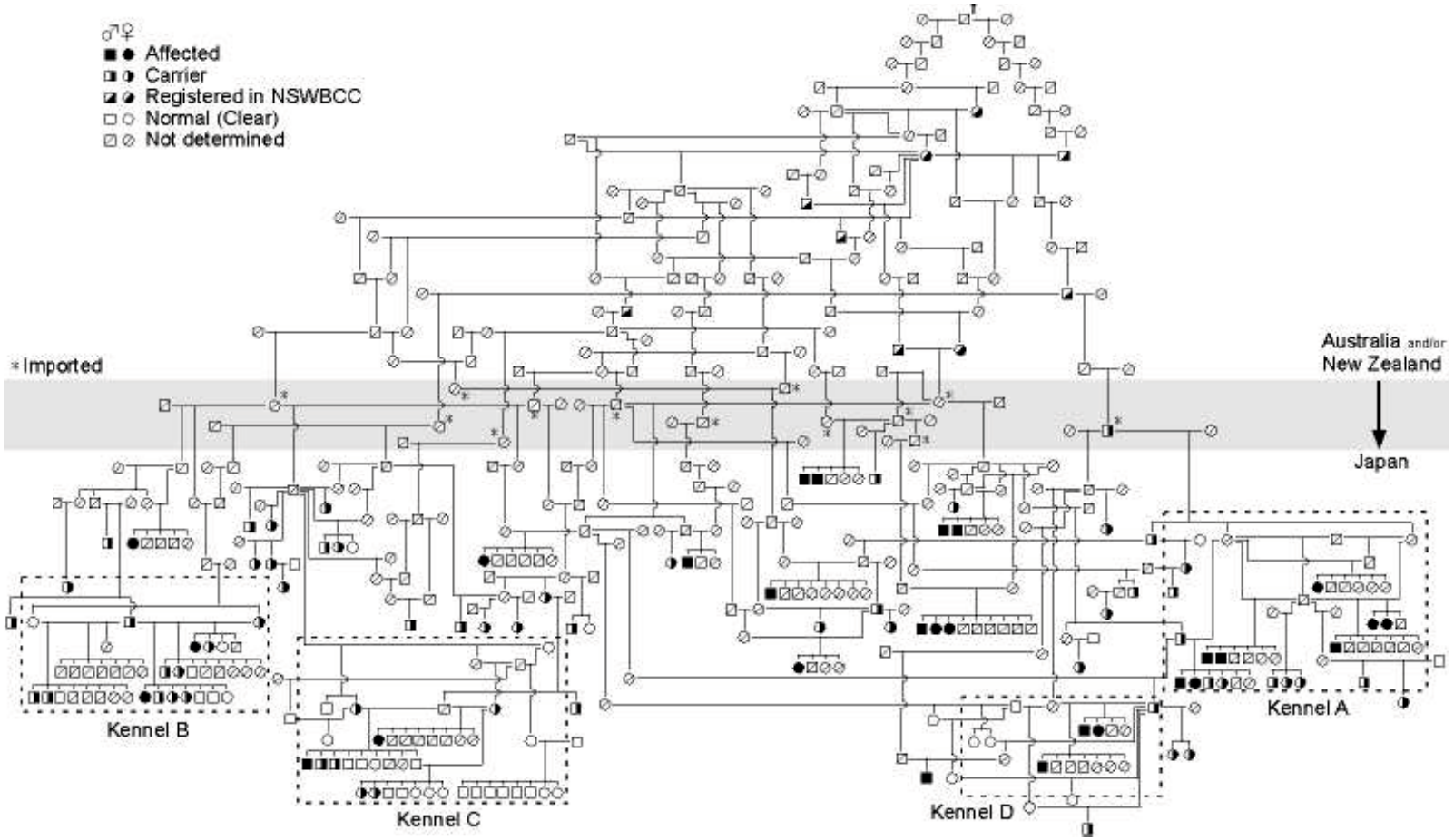

Figure 9. Genetic relationship of affected and carrier dogs of canine neuronal ceroid lipofuscinosis identified in Japan between 2000 and 2013. The analysis was carried out using pedigree papers issued by the Japan Kennel Club and the Kennel Club of Japan and information reported by the New South Wales Border Collie Club (NSWBCC) and the Border Collie Database. The gray area indicates a border between Oceania and Japan. Dogs imported from Oceania to Japan were marked with an asterisk (*). All dogs carrying the mutant allele shared a common ancestor (†) born in 1944 in Australia. Areas surrounded by a dashed line indicate the 4 kennels surveyed using a genotyping test.

CHAPTER 3

Clinical study, rapid diagnostic assays and frequency of the mutant allele of trapped neutrophil syndrome in Border Collies

The above-titled work originally appeared in "*The Journal of Veterinary Medical Science* (Mizukami et al., 2011)" and "*The Veterinary Journal* (Mizukami et al., 2012)" as: ***Trapped Neutrophil Syndrome in a Border Collie Dog: Clinical, Clinico-pathologic, and Molecular Findings*** and ***Real-time PCR genotyping assay for canine trapped neutrophil syndrome and high frequency of the mutant allele in Border collies*** authored by:

The Journal of Veterinary Medical Science

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3.1. ABSTRACT

Trapped neutrophil syndrome (TNS) is an autosomal recessive inherited neutropenia known in Border Collies since the 1990's. Recently, the causative mutation has been identified in the canine *VPS13B* gene and a DNA-based diagnosis has now become available. The present paper describes clinical and clinico-pathologic findings in a Border Collie with TNS that was molecularly diagnosed for the first time in Japan. In a 10-week-old male Border Collie with microgenesis and symptoms related to recurrent infections, a hematological examination revealed severe leukopenia due to neutropenia, suggesting the dog to be affected by inherited neutropenic immunodeficiency. Direct DNA sequencing demonstrated that the dog was homozygous for the causative mutation of TNS and both its parents were heterozygous carriers. In addition, a polymerase chain reaction (PCR)-based length polymorphism analysis coupled with microchip electrophoresis and a real-time PCR assay with TaqMan minor groove binder probes were developed for the genotyping of TNS. A genotyping survey was carried out in Border collies in Japan. The carrier frequency was 11.1%, suggesting that the mutant allele frequency is high enough to warrant measures to control and prevent the disease.

3.2. INTRODUCTION

An autosomal recessive inherited neutropenia, currently called trapped neutrophil syndrome (TNS), has been known in the Border Collie breed (Allan et al., 1996: Shearman and Wilton, 2011). The disease, originally detected in Border Collies in Australia and New Zealand in the 1990's, is characterized by a marked reduction in numbers of neutrophils in peripheral blood and a hyperplasia of myeloid cells in bone marrow (Allan et al., 1996). Due to the severe neutropenia, affected dogs are consistently subject to life-threatening infections, resulting in premature death. However, a definitive diagnosis has been difficult because the original clinical characteristics except neutropenia have yet to be completely determined and the clinical features are often modified by chronic and recurrent infections.

Recently, the causative mutation of TNS has been identified as a 4-base pair (bp) deletion in exon 19 of the canine *VPS13B* gene (g.4411950_4411953delGTTT), resulting in the premature truncation of the gene product and a carrier frequency of 14/83 (16.9%) was demonstrated in Border collies in Japan (Shearman and Wilton, 2011). The *VPS13B* gene encodes a potential transmembrane protein involved in vesicle-mediated transport and sorting within the cell, defects of which cause Cohen syndrome in humans (Kolehmainen et al., 2003). There is a strong similarity in clinical features such as neutropenia between human Cohen syndrome and canine TNS (Shearman and Wilton, 2011), suggesting TNS to be a valuable model for Cohen syndrome. Furthermore, the identification of the causative mutation for TNS has now provided veterinary practitioners a means of achieving a definitive diagnosis and enabled subsequent characterization of the original features of canine TNS.

The present report describes clinical and clinico-pathologic findings in a Border Collie

pup with TNS that was molecularly diagnosed for the first time in Japan. In addition, a simple and rapid polymerase chain reaction (PCR)-based length polymorphism (LP) analysis coupled with microchip electrophoresis and a real-time PCR with TaqMan minor groove binder (MGB) probes were developed for rapid genotype discrimination and a genotyping survey was carried out in a population of Border collies in Japan to determine the allele frequency.

3.3. CASE PRESENTATION

A 10-week-old male Border Collie weighing 3.5 kg with a black and white coat was presented to a private veterinary hospital because of decreased activity, anorexia, fever (40.5°C/104.9°F), vomiting, and polyuria/polydipsia. The dog showed microgenesis without dysmorphism, a decreased level of consciousness, astasia, and incontinence. Postural reactions including proprioception and placing and hopping reflexes were markedly reduced, but skin reflex and deep pain sense were normal. The dog had been treated for intestinal coccidiosis 6 days before and for vomiting and diarrhea of unknown cause 10 days before in different veterinary hospitals. The dog had responded to each symptomatic treatment and its condition had improved temporarily each time.

A complete blood count was carried out using an automated hematocytometer (pocH-100i V Diff, Sysmex Corp., Kobe, Japan) capable of measuring a preliminary leukogram composed of lymphocytes, eosinophils, and other types of cells such as neutrophils and monocytes (Table 5). The total concentration of protein in serum was determined by a protein refractometer and other serum chemistry results were obtained using a blood chemistry autoanalyzer (Fuji Dri-Chem 3500V, Fujifilm Corp., Tokyo, Japan). Unfortunately, no examinations of blood and bone marrow aspirate smears were performed. Results of the clinico-pathological examinations are shown in Table 5. A marked decrease in the leukocyte count was observed and severe neutropenia was strongly suggested by the automated differential counts. Mild non-regenerative anemia was suggested by slight decreases in the erythrocyte count, hemoglobin concentration, and hematocrit value with no changes in erythrocyte indices. Among the blood chemistry results, decreases in glucose, creatinine,

sodium, potassium, and chloride and increases in alkaline phosphatase (ALP) and inorganic phosphorus (iP) were observed. Canine distemper virus and canine parvovirus were not detected using commercial tests (Checkman CDV and Checkman CPV, Adtec, Ohita, Japan). No abnormality was detected in routine fecal, urinary, ophthalmologic, and X-ray examinations.

The dog died the next day without responding to symptomatic treatments including antibiotic and fluids, but a necropsy was not performed. The cause of death might be a septic shock. Based on the signalment and laboratory data, the dog was suspected of having an inherited neutropenic immunodeficiency such as TNS and cyclic neutropenia (CN) which is also known as gray collie syndrome (Benson et al., 2003; Dale et al., 1972; Meng et al., 2010). Therefore, a molecular analysis for the diagnosis of these diseases was performed.

A blood specimen was obtained from the affected dog and spotted onto Flinders Technology Associates filter paper (FTA card; FTA Classic Card, Whatman International Ltd., Piscataway, NJ, U.S.A.). Saliva specimens were collected from the clinically healthy sire and dam of the affected dog using FTA cards (Indicating FTA Classic Card, Whatman International Ltd.). Blood samples from four healthy Beagles, stored using FTA cards, were used as a control. A disc punched out of a blood- or saliva-spotted FTA card was used directly as a template for PCR after quick washing as reported previously (Mizukami et al., 2011, 2012b).

Direct DNA sequencing was carried out to confirm the presence or absence of the TNS- and CN-causative mutations; a 4-bp deletion in exon 19 of the canine *VPS13B* gene (Shearman and Wilton, 2011) and an insertion of an extra adenine residue within a tract of 9

adenines in exon 21 of the canine *AP3B1* gene (Benson et al., 2003), respectively. To amplify the sequences around these causative mutations, 2 sets of oligonucleotide primers (Table 6) were designed based on GenBank data (reference nos. NC_006595 and NC_006585, respectively). PCR amplification was performed in a 20-μl reaction mixture containing 10 μl of 2× PCR master mix (GoTaq Hot Start Green Master Mix, Promega Corp., Madison, WI, U.S.A.), 12.5 pmol of forward and reverse primers and a treated FTA disc as a template. After the first denaturation at 94°C for 2 min, 40 cycles of amplification were carried out, at a denaturing temperature of 94°C for 1 min, an annealing temperature of 55°C for 1 min, and an extension temperature of 72°C for 1 min. Extension during the last cycle was carried out for 2 min. The PCR products were subjected to electrophoresis in a 3% (wt/vol) agarose gel, stained with ethidium bromide, and visualized under an ultraviolet transilluminator. Amplified DNA fragments were extracted from the gel using a commercial kit (QIAquick Gel Extraction Kit, Qiagen, Tokyo, Japan). Direct DNA sequencing of the purified PCR product was carried out by a commercial company (Hokkaido System Science Co., Ltd., Sapporo, Japan).

Direct DNA sequencing demonstrated that the affected dog was homozygous for the causative mutation of TNS and its parents were both heterozygous carriers (Fig. 10), indicating that the dog was affected by TNS and inherited a mutant allele from each of the parents. The sequencing also demonstrated that the dog was not affected by CN, being homozygous for the wild-type 9 adenines in the canine *AP3B1* gene (Fig. 11). This is the first case of TNS to be molecularly diagnosed in Japan.

3.4. MATERIALS AND METHODS

Control samples for each genotype were obtained from an affected Border collie (homozygous for the mutant allele) and the sire and dam of the affected dog (heterozygous carriers) previously genotyped by direct DNA sequencing (Mizukami et al., 2012d). Blood samples from four healthy Beagles (homozygous for the wild-type allele) were used as a control after confirmed their genotypes by direct DNA sequencing. DNA templates for the LP analysis and the real-time PCR assay were prepared using whole blood or saliva spotted onto FTA cards (FTA classic card, Whatman International Ltd, Piscataway, NJ.). A disc punched out of these cards was used for DNA template directly or after extraction (Chang et al., 2010a; Mizukami et al., 2011, 2012b). The LP analysis was conducted with the forward and reverse primers listed in Table 6 using the same protocol and reagents as for direct DNA sequencing. The analysis of the products was performed using a microchip electrophoresis system (MCE-202 MultiNA Microchip Electrophoresis System, Shimadzu Corp., Kyoto, Japan), as reported previously (Mizukami et al., 2011, 2012b). Real-time PCR amplification was conducted with the primers and probes (Table 6) using the same protocol and reagents described previously (Mizukami et al., 2012b).

The genotyping survey was carried out using DNA samples from 469 Border collies in Japan aged 3 months to 14 years. These samples were collected with the owners' informed consent by the Japan Border Collie Health Network, a volunteer breeders' association, from 2006 to 2013.

3.5. RESULTS

The LP analysis revealed a 60-bp band for the wild-type allele and a 56-bp band for the mutant allele, which occurred at distinctly different sites on the gel image following microchip electrophoresis, allowing easy discrimination of the 3 genotypes (Fig. 12). The real-time PCR assay with TaqMan MGB probes also determined all genotypes of canine TNS without non-specific allelic amplification after 50 cycles of amplification (Fig. 13). The total required time for 50 cycle amplification was approximately 40 min. An allelic discrimination plot was constructed based on the three genotypes of amplification plots obtained using DNA samples from seven animals, including one affected, two carrier and four wild-type dogs (Fig. 14). In the genotyping survey, 52/469 (11.1%) Border collies in Japan were carriers; no homozygous (affected) dogs were identified.

3.6. DISCUSSION

To our knowledge, only two reports have described TNS in Border Collies (Allan et al., 1996; Mizukami et al., 2011). One case report in New Zealand 15 years ago described in some detail the clinical features of two littermates suspected of having inherited neutropenia, but not molecularly diagnosed with TNS (Allan et al., 1996). The other report, a recent paper demonstrating a causative mutation, provided some information on clinical features in some affected dogs molecularly diagnosed with TNS (Shearman and Wilton, 2011). According to these reports, affected pups are often smaller than healthy littermates and sometimes show abnormal craniofacial development with a narrowed and elongated skull described by breeders as ferret-like (Shearman and Wilton, 2011). Furthermore, they suffer from chronic or recurrent infections and fail to thrive resulting from a compromised immune system (Allan et al., 1996). Clinical signs include dullness, depression, fever, awkward gait followed by astasia, decreased postural reaction, diarrhea, and osteomyelitis of the femur and tibia (Allan et al., 1996). In the present study, a 10-week-old affected pup with an approximately 2-week history of recurrent infections showed clinical features as follows: microgenesis (small body size) and infection-related signs including fever, vomiting, diarrhea, anorexia, and decreased level of consciousness. Considering the clinical features reported previously (Allan et al., 1996: Shearman and Wilton, 2011), microgenesis and signs related to chronic and recurrent infections may be common traits, but their onset, degree, and variation seem to depend on environmental factors such as colostrum intake and surrounding microorganisms.

Regarding clinico-pathologic features, Allan et al. (1996) reported information on two affected littermates: hematologically, severe neutropenia along with a severe degenerative left

shift accompanying metamyelocytes and myelocytes; marked monocytosis; eosinopenia; mild non-regenerative anemia with increased circulating nucleated erythrocytes and unusual erythrocyte morphology in peripheral blood; and hyperplasia of myeloid cells with degeneration of neutrophil series cells (Allan et al., 1996). In terms of serum chemistry, increases in ALP, iP and cholesterol and decreases in creatinine and albumin were observed in the two dogs. In the present study, marked leukopenia due to severe neutropenia, mild non-regenerative anemia, increases in ALP and iP, and decreases in creatinine, glucose, and electrolytes were observed in the affected dog (Table 5). Common significant features may be leukopenia due to neutropenia, non-regenerative anemia, and decreased concentration of creatinine. The leukopenia and neutropenia are likely a primary change in TNS. The non-regenerative anemia may be secondary due to the occupancy of erythropoietic areas by hyperplastic myeloid series cells or inhibition of erythropoiesis by chronic inflammatory mediators. Decreased creatinine concentration may be induced secondarily by reduced muscle metabolism resulting from growth retardation. Other changes in the serum chemistry seem to depend on aging, individual body conditions, and secondary clinical symptoms. However, to characterize the clinical and clinio-pathologic features of canine TNS, more studies are required using information obtained from a number of dogs molecularly diagnosed with a genetic test.

It is quite difficult to diagnose TNS using only clinical and clinico-pathologic data because these data can be modified easily by a variety of infectious and inflammatory conditions. In general, canine neutropenia arises from many causes other than TNS and CN, i.e., myelophthisis, certain drugs, toxins, infections, endotoxin shock, anaphylactic shock,

immune-mediated disorders, and paraneoplastic syndrome (Couto, 2008). Therefore, a definitive diagnosis of TNS requires a genetic test for the differentiation of these disorders.

In addition, to prevent and eradicate this fatal hereditary disease, the determination of genotypes through systematic monitoring and the continuous removal of carriers from breeding colonies would be among the most important and efficient measures, requiring a simple, rapid, and inexpensive genetic test. Actually, the frequency of the mutant allele of TNS seems high enough that measures to control and prevent the disease would be warranted. The carrier frequency of TNS among Border collies in the present study was 11.1%, which is lower than 16.9% reported previously in Japan by Shearman and Wilton (2011). This difference may be related to the larger sample size in the present study. Shearman and Wilton (2011) also reported high carrier frequencies among Border collies in the USA (16.5%), Australia (15.4%), Finland (13.0%), Germany (12.9%), the UK (11.3%) and the Netherlands (8.1%). The carrier frequency for TNS in Border collies in Japan is high compared to that for other fatal canine genetic diseases, such as neuronal ceroid lipofuscinosis in Border collies (7.0% in Chapter 1) and GM1 gangliosidosis in Shibas (2.9%; Yamato et al., 2008). Given that nearly 4000 Border collies are registered in the Japan Kennel Club annually (http://www.jkc.or.jp/), dozens of affected dogs may be born in Japan in the course of a year. The availability of rapid genotyping assays developed in the present study should assist with prevention and control of this genetic disease as well as individual diagnosis.

Table 5. Results of clinico-pathological examinations in the affected dog.

Subjects (units)	Affected dog	Reference range*
Complete blood count:		
Leukocytes ($\times 10^3/\mu l$)	2.2	10.9–19.2
Lymphocytes (/μl)	900	1949–8169
Eosinophils (/μl)	400	0–827
Others** (/μl)	900	5363–14077
Erythrocytes ($\times 10^6/\mu l$)	4.46	4.37–6.53
Hemoglobin (g/dl)	9.8	10.2–13.4
Hematocrit (%)	28.5	31.4–43.0
MCV (fl)	63.9	62.8–74.4
MCH (pg)	22.0	18.8–24.8
MCHC (g/dl)	34.4	29.2–34.4
Platelets ($\times 10^3/\mu l$)	329	200–500
Serum chemistry:		
Total protein (g/dl)	6.0	5.0–7.2
Albumin (g/dl)	2.6	2.6–4.0
Glucose (mmol/l)	3.7	4.1–7.0
Alanine aminotransferase (U/l)	25	17–78
Aspartate aminotransferase (U/l)	23	17–44
Alkaline phosphatase (U/l)	948	69–333
Total cholesterol (mmol/l)	7.36	2.89–8.11
Total bilirubin (μmol/l)	5.13	1.71–8.55
Ammonia (μmol/l)	38.8	9.4–44.0
Blood urea nitrogen (mmol/l)	10.7	3.3–10.4
Creatinine (μmol/l)	17.7	35.4–124
Calcium (mmol/l)	2.93	2.33–3.03
Inorganic phosphorus (mmol/l)	3.39	2.62–3.13
Sodium (mmol/l)	135	141–152
Potassium (mmol/l)	3.6	3.8–5.0
Chloride (mmol/l)	96	102–117

* Values were cited from a textbook (Schalm, 1986) for the complete blood count (CBC), and from another textbook (Meyer and Harvey, 1998) and the data provided by the maker of a blood chemistry autoanalyzer (Fuji Dri-Chem 3500V, Fujifilm Corp., Tokyo, Japan) for serum chemistry. Reference ranges for inorganic phosphorus, alkaline phosphatase, and CBCs except platelets were the data applicable for dogs at 8 weeks (Meyer and Harvey, 1998), 1 year old and younger (Fujifilm Corp.), and 9–12 weeks of age (Schalm, 1986), respectively. Ranges are the mean ± 2 standard deviations.

** The number is composed of neutrophil and monocyte counts, which was calculated using an automated hematocytometer (pocH-100i V Diff, Sysmex Corp., Kobe, Japan).

Table 6. Characteristics of primers and TaqMan probes used in the present study.

Name*	Assay*	Primer/probe	Sequence 5'→3' (mer)	Location on genome†	Tm (°C)	Concentration (nM)
TNS-DS-F	DS for TNS	Forward	GTCCTACTTGGAGTGAAGTG (20)	4411801–4411820 in Chr. 13	56.3	625
TNS-DS-R	DS for TNS	Reverse	AGGACTTGTGGTACATCCAT (20)	4412081–4412100 in Chr. 13	54.3	625
CN-DS-F	DS for CN	Forward	GGAAGGCTAAGTGGAGCAAA (20)	31550526–31550545 in Chr. 3	56.3	625
CN-DS-R	DS for CN	Reverse	AGGTCCATGCTCTCTCTTAC (20)	31550764–31550783 in Chr. 3	56.3	625
LP-F	LP analysis	Forward	AATATTGACCCAGTCTTA (18)	4411920–4411937 in Chr. 13	46.7	625
LP-R	LP analysis	Reverse	TCTACTGGTTCGTTTCTG (18)	4411962–4411979 in Chr.13	51.2	625
RT-F	Real-time PCR	Forward	GTAGCGTACAAGGAGTAGCAGTTAA (25)	4411897-4411921 in Chr.13	56.1	450
RT-R	Real-time PCR	Reverse	CTGCATATGTCTACTGGTTCGTTTC (25)	4411964-4411988 in Chr.13	55.6	450
Probe-VIC-W	Real-time PCR	Probe for wild allele	CTGGCTTGTTTATCAGCC (18)	4411943-4411960 in Chr.13	–	100
Probe-FAM-M	Real-time PCR	Probe for mutant allele	AACTGGCTTATCAGCC (16)	4411941-4411949, 4411954-4411960 in Chr.13	–	100

* TNS = trapped neutrophil syndrome; CN = cyclic neutropenia; DS = direct DNA sequence; LP = length polymorphism; F = forward; R = reverse. † The sequence and number are based on the GenBank information (reference nos. NC_006595 and NC_006585 for TNS and NC, respectively). Chr. = chromosome.

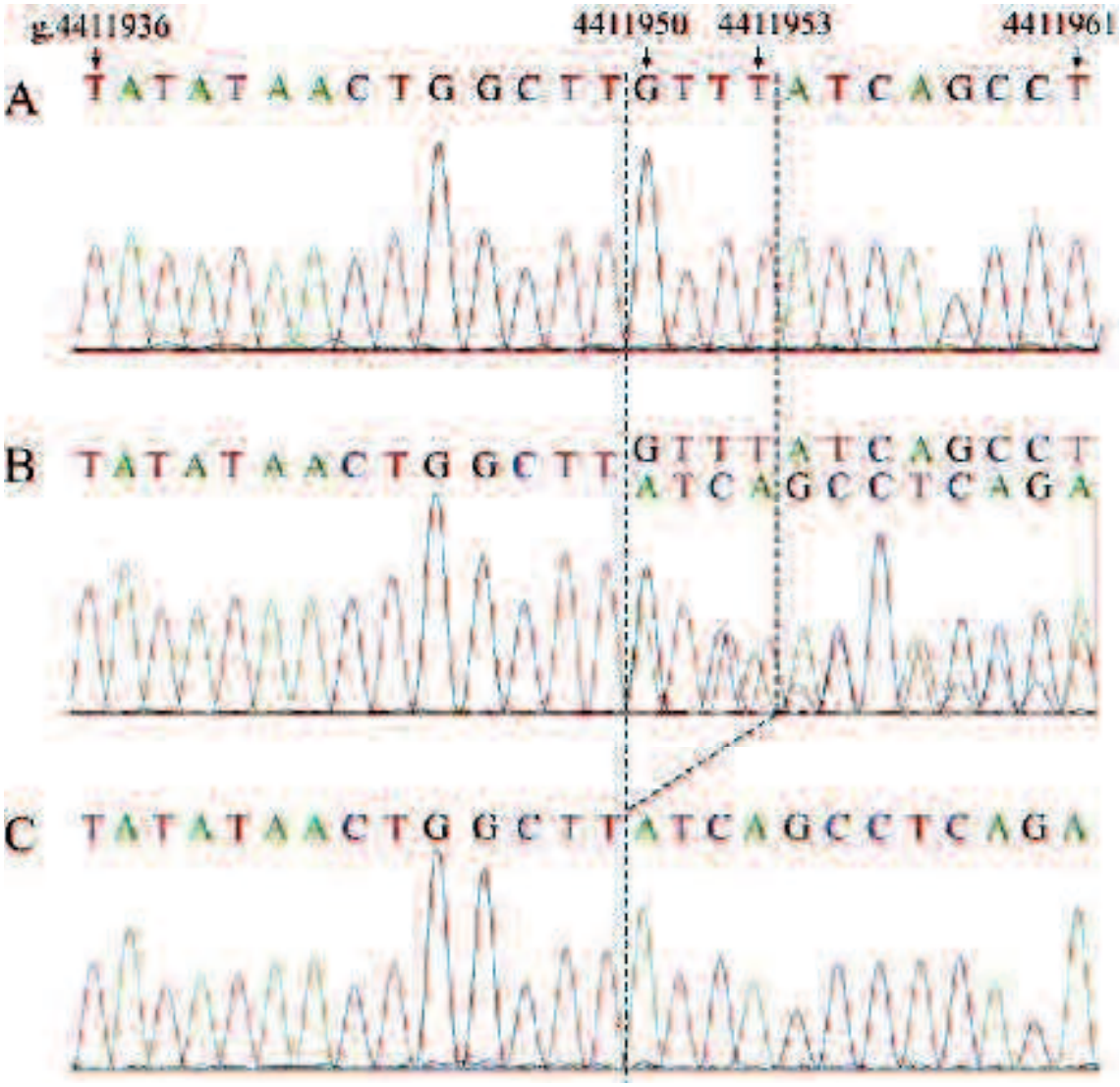

Figure 10. Electropherograms of exon 19 of the canine *VPS13B* gene from a wild-type dog (A; an unaffected Beagle), a heterozygous carrier (B; the sire of the affected dog), and the affected dog (C; a Border Collie with trapped neutrophil syndrome). The carrier was heterozygous for the 4-base pair (GTTT) deletion and the affected dog, homozygous. The deletion is not detected in the unaffected dog.

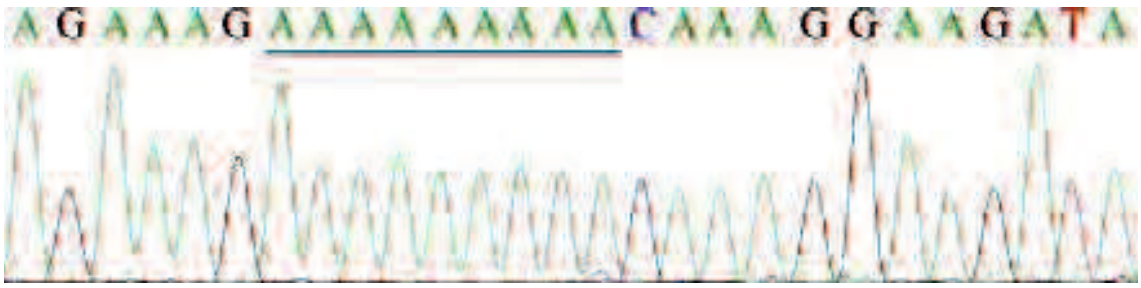

Figure 11. An electropherogram of exon 21 of the canine *AP3B1* gene from a Border Collie with trapped neutrophil syndrome. The cyclic neutropenia-related mutation, an insertion of an extra adenine within a tract of 9 adenines, is not detected demonstrating a homozygous wild-type sequence (9 adenines) in this region (underline).

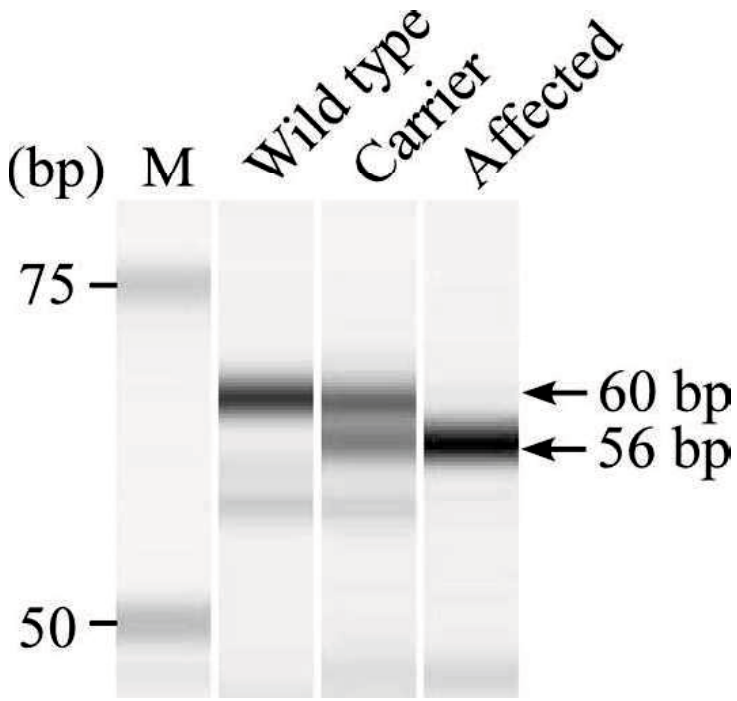

Figure 12. Electrophoretograms of the microchip using the length polymorphism analysis for canine trapped neutrophil syndrome. Fragment patterns in the 3 genotypes, the wild-type, heterozygous carrier, and affected dog are shown with molecular size markers (M). bp: base pair.

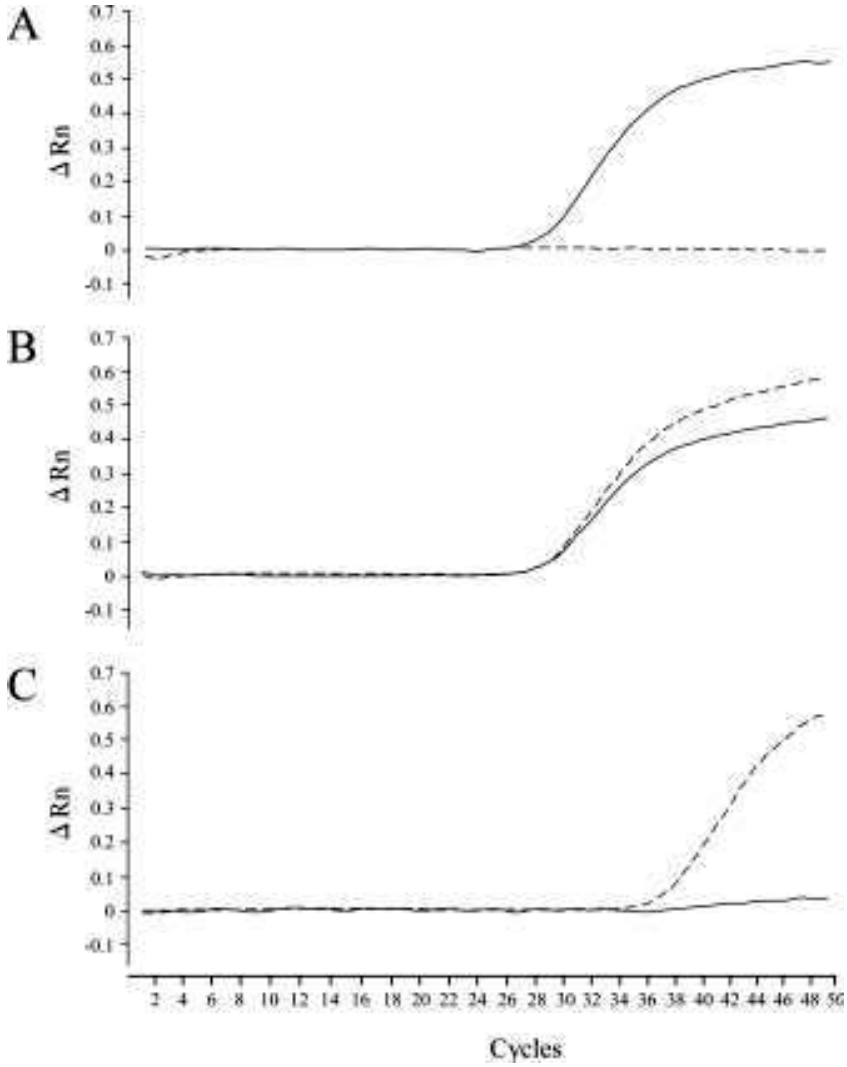

Figure 13. Real-time PCR amplification plots of wild-type and mutant alleles in canine trapped neutrophil syndrome. Amplification was plotted as fluorescence intensity (ΔRn value) against cycle number. The ΔRn value is the reporter dye signal normalised to an internal reference dye and corrected for the baseline signal established in the first few cycles of the reaction. Each of three amplification plots showed the wild-type (A), heterozygous carrier (B), and homozygous affected dogs (C). Solid and dotted lines indicate wild-type and mutant alleles, respectively.

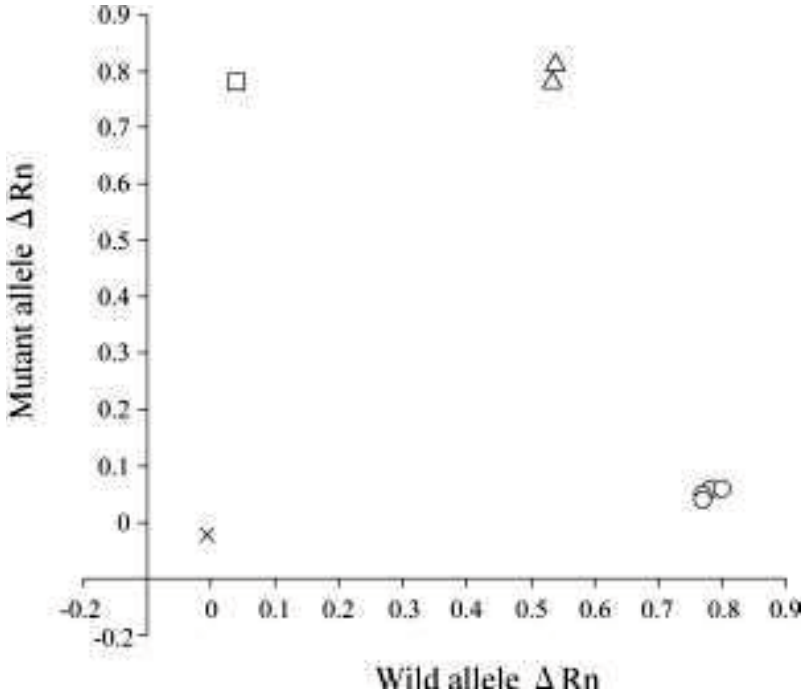

Figure 14. Allelic discrimination plot of end-point fluorescence real-time PCR data showing the three genotypes of trapped neutrophil syndrome. The plot is depicted using seven representative DNA samples already genotyped by direct DNA sequencing. The plot is expressed as fluorescence intensity (ΔRn value) for each allele along the x- and y-axes. The ΔRn value in this figure is the end-point reporter dye signal normalised to an internal reference dye and corrected for the baseline signal established in the first few cycles of the reaction. ×, No template control; ○, wild-type (4 samples); Δ, carrier (2 samples); □, affected genotypes (1 sample).

CHAPTER 4.1

Rapid diagnostic assays for the 4-base pair deletion of canine *MDR1/ABCB1* gene and frequency of the mutant allele in Border Collies

The above-titled work originally appeared in "*Journal of Veterinary Diagnostic Investigation* (Mizukami et al., 2012)" as: ***Rapid genotyping assays for the 4–base pair deletion of canine MDR1/ABCB1 gene and low frequency of the mutant allele in Border Collie dogs*** authored by:

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4.1.1. ABSTRACT

P-glycoprotein, encoded by the *MDR1*/*ABCB1* gene, is an integral component of the blood–brain barrier as an efflux pump for xenobiotics crucial in limiting drug uptake into the central nervous system. Dogs homozygous for a 4–base pair deletion of the canine *MDR1*/*ABCB1* gene show altered expression or function of P-glycoprotein, resulting in neurotoxicosis after administration of the substrate drugs. In the present study, the usefulness of microchip electrophoresis for genotyping assays detecting this deletion mutation was evaluated. Mutagenically separated polymerase chain reaction (MS-PCR) and real-time PCR assays were newly developed and evaluated. Furthermore, a genotyping survey was carried out in a population of Border Collies dogs in Japan to determine the allele frequency in this breed. Microchip electrophoresis showed advantages in detection sensitivity and time saving over other modes of electrophoresis. The MS-PCR assay clearly discriminated all genotypes. Real-time PCR assay was most suitable for a large-scale survey due to its high throughput and rapidity. The genotyping survey demonstrated that the carrier and mutant allele frequencies were 0.4% and 0.2%, respectively, suggesting that the mutant allele frequency in Border Collies is markedly low compared to that in the susceptible dog breeds such as rough and smooth Collies.

4.1.2. INTRODUCTION

P-glycoprotein (P-gp), an adenosine triphosphate (ATP)-driven multidrug efflux carrier, is the product of the multidrugresistance *MDR1*/*ABCB1* gene (Fromm, 2004). More recently, the *MDR1*/*ABCB1* gene has also been referred to as *ABCB1* based on the systematic nomenclature of the ATP-binding cassette transporter family (Dean, 2005). P-glycoprotein is expressed in various mammalian tissues such as the brush border membrane of epithelial cells in the intestinal tract, the luminal membrane of proximal tubules in the kidney, and the canalicular membrane of liver hepatocytes (Thiebaut et al., 1987). Apical and luminal expression of P-gp in these organs diminishes oral drug bioavailability and promotes drug elimination into urine and bile (Fromm, 2000). Additionally, at the blood–brain barrier, P-gp is expressed at the luminal membrane of endothelial cells of the brain capillaries (Cordon-Cardo et al., 1989) and restricts the entry of drugs into the central nervous system by an efflux-based transport mechanism (Schinkel, 1997).

In 2001, a 4–base pair (bp) deletion mutation (c.227_230del) associated with ivermectin toxicosis (IT) was identified in the canine *MDR1*/*ABCB1* gene and was referred to as *mdr1-1Δ*, *ABCB1-1Δ*, or *MDR1* nt230(del4) (Geyer et al., 2005a; Mealey et al., 2001, 2008; Neff et al., 2004). This mutation results in generation of a truncated P-gp with a length of approximately 7% of the full-length P-gp (Roulet et al., 2003). The *MDR1*/*ABCB1* mutation correlates with the ivermectin-sensitive phenotype that was recognized in Collie dogs in the early 1980s (Pulliam et al., 1985; Seward, 1983). To date, this mutation has been detected in more than 10 dog breeds including rough Collie, smooth Collie, Shetland Sheepdog, Australian Shepherd, miniature Australian Shepherd, Border Collie, Old English Sheepdog,

English shepherd, German Shepherd Dog, white Swiss Shepherd, Wäller, longhaired Whippet, Silken Windhound, and McNab (Fecht and Distl, 2008; Geyer et al., 2005b; Gramer et al., 2011; Mealey et al., 2008b; Neff et al., 2005). Dogs homozygous for the deletion mutation do not express a functionally intact P-gp and show increased sensitivity to many P-gp–transported drugs such as moxidectin, milbemycin oxime, acepromazine, butorphanol, digoxin, vincristine, and loperamide as well as ivermectin (Fecht and Distl, 2008; Martinez et al., 2008; Mealey et al., 2008a). There is also a report that the pharmacokinetic parameters of fexofenadine and quinidine are significantly altered in *MDR1*/*ABCB1* mutant dogs. Regarding some of these drugs, brain penetration is highly increased, and the dogs develop severe neurotoxicosis, even when the drugs are administered at a normal therapeutic dosage, as in the case of ivermectin and doramectin (Fecht and Distl, 2008; Martinez et al., 2008; Mealey et al., 2008a).

Several different genotyping methods have been described for the detection of the 4-bp deletion mutation (Baars et al., 2008; Geyer et al., 2005a; Kawabata et al., 2005; Klintzsch et al., 2010; Neff et al., 2004; Roulet et al., 2003). Most of these methods use PCR amplification of the flanking regions followed by length polymorphism (LP) analysis most often performed by polyacrylamide gel electrophoresis (PAGE) (Geyer et al., 2005a; Neff et al., 2004; Roulet et al., 2003) and rarely by agarose gel electrophoresis (Kawabata et al., 2005). These methods have various problems including time-consuming technique, detection sensitivity, and waste management. In the present study, the usefulness of microchip electrophoresis for LP-based genotyping assays was evaluated. Mutagenically separated (MS)-PCR and real-time PCR assays were newly developed for clear genotype discrimination. Furthermore, the genotyping

survey was carried out in a population of Border Collies in Japan to determine the allele frequency in this breed.

4.1.3. MATERIALS AND METHODS

Standard samples and treatment

Control DNA samples for each genotype were obtained from 7 rough and 2 smooth Collies from a kennel specializing in Collies after obtaining informed consent from the breeder, and 4 Beagles kept as blood donors by the Veterinary Teaching Hospital in Kagoshima University. The samples were collected as saliva specimens spotted on Flinders Technology Associates filter papers (FTA card; Indicating FTA Classic Card; Whatman International Ltd., Piscataway, NJ.), using a special applicator (Sterile Foam Tipped Applicator, Whatman International Ltd., Piscataway, NJ.) to swab saliva, and stored at approximately 4ºC until used. The genotypes of these dogs were determined by direct DNA sequence analysis by a commercial company (Hokkaido System Science Co. Ltd., Sapporo, Japan.) using the primer pair DS-F and DS-R shown in Table 7, demonstrating 6 mutant homozygous (–/–) Collies (5 rough and 1 smooth), 3 heterozygous carrier (+/–) Collies (2 rough and 1 smooth), and 4 normal homozygous (+/+) Beagles. For a DNA template of each PCR such as direct DNA sequence, LP analysis, and MS-PCR assay, a 1.2-mm in diameter disc punched from the FTA card using a hole punch (Harris Uni-Core Punch (size 1.2 mm), Whatman International Ltd., Piscataway, NJ.) was used. The disc was placed into a separate 0.2-ml tube for PCR. The disc on the tube bottom was washed 3 times for 5 min with 100 µl of washing solution (FTA Purification Reagent; Whatman International Ltd., Piscataway, NJ.), rinsed twice for 5 min with 200 µl of Tris–ethylenediamine tetra-acetic acid buffer (pH 8.0)

(10× TE Powder, pH 8.0, Wako Pure Chemical Industries Ltd., Osaka, Japan.), and dried at 60ºC for 10 min. The treated disc was used directly as a template for these PCR procedures, except real-time PCR.

LP analysis method

The LP analysis was carried out with forward (LP-F) and reverse (LP-R) primers reported previously (Kawabata et al., 2005) in a 20 μl reaction mixture containing 10 μl of 2× PCR master mix (GoTaq Hot Start Green Master Mix, Promega Corp., Madison, WI.), 12.5 pmol of primers, and the treated disc of FTA card as a template. After the first denaturation at 94ºC for 2 min, 40 cycles of amplification were carried out, at a denaturing temperature of 94ºC for 1 min, an annealing temperature of 56.2ºC for 1 min, and an extension temperature of 72ºC for 1 min. Extension during the last cycle was carried out for 2 min.

MS-PCR method

The MS-PCR is a method utilizing sequence-specific hybridization by 2 primers with widely different sizes in 1 PCR tube without a restriction endonuclease digestion process (Roulet et al., 2003). In the present study, the MS-PCR assay was designed based on the general rules of MS-PCR (Roulet et al., 2003), and the ratio of short to long allele-specific forward primers (MS-W-F and MS-M-F) was increased to 10:1 on a molar basis (Table 7) in order to produce bands of similar intensity in the heterozygous carrier when visualized on

electrophoresis. The MS-PCR assay was conducted using these 2 allele-specific forward primers and a reverse primer (MS-R) simultaneously in a 20-μl reaction mixture containing 10 μl of 2× PCR master mix (GoTaq Hot Start Green Master Mix, Promega Corp., Madison, WI.), 1.25 pmol of the MS-W-F primer, 12.5 pmol of MS-M-F and MS-R primers, and the treated disc of FTA card as a template. After the first denaturation at 95ºC for 5 min, 40 cycles of amplification were carried out, at a denaturing temperature of 95ºC for 30 sec, an annealing temperature of 56ºC for 45 sec, and an extension temperature of 72ºC for 45 sec. Extension during the last cycle was carried out at 72ºC for 3 min 45 sec.

Electrophoresis

The products of LP analysis and MS-PCR assay were subjected to electrophoresis on 5% and 3% (wt/vol) agarose gel (Agarose 21, Nippon Gene Co. Ltd., Tokyo, Japan.), respectively. The product of LP analysis was also subjected to electrophoresis on 20% (wt/vol) polyacrylamide gel (20% TBE Gel, Invitrogen Corp., Carlsbad, CA.). Molecular size markers (25/100 bp Mixed DNA Ladder, Bioneer, Daejeon, Korea) were used in both electrophoreses. The electrophoresed agarose gel was stained with ethidium bromide and irradiated by an ultraviolet transilluminator. The polyacrylamide gel was stained with a silver staining kit (SilverXpress Silver Staining Kit, Invitrogen Corp., Carlsbad, CA.). Analysis of PCR products was also performed using a microchip electrophoresis system (MCE-202 MultiNA Microchip Electrophoresis System, Shimazu Corp., Kyoto, Japan.) with a special reagent kit (DNA-500 Kit, Shimazu Corp., Kyoto, Japan.) that included internal DNA size markers and

DNA separation buffer. A fluorescent dye (SYBR Gold Nucleic Acid Gel Stain, Invitrogen Corp., Carlsbad, CA.) was added to the DNA separation buffer according to the manufacturer's protocol for the microchip electrophoresis system. DNA ladder markers (25 bp DNA Ladder, Invitrogen Corp., Carlsbad, CA.) from 25 to 450 bp were used as references for DNA sizing.

Real-time PCR method

For a DNA template of real-time PCR, a 1.2-mm in diameter disc was punched from the aforementioned FTA cards using a hole punch (Harris Uni-Core Punch (size 1.2 mm), Whatman International Ltd., Piscataway, NJ.), placed into a separate 0.2-ml tube, lysed in the tube with 8 μl of lysis solution from a special DNA extraction kit (DNA Extract All Lysis Reagents Kit, Applied Biosystems, Foster City, CA.), and subsequently incubated at 95ºC for 3 min. Then, 8 μl of DNA stabilizing solution from the kit (DNA Extract All Lysis Reagents Kit, Applied Biosystems, Foster City, CA.) was added to the tube. This DNA-containing solution was transferred to a new tube and stored at –25ºC until analysis. Amplifications were performed on a real-time PCR system (StepOne Real-Time PCR System, Applied Biosystems, Foster City, CA.) using a specific primer pair (RT-F and RT-R) and TaqMan minor groove binder (MGB) probes bound with each fluorescent reporter dye (6-carboxyrhodamine or 6-carboxyfluorescein) at the 5'-end and a nonfluorescent quencher dye at the 3'-end (Table 7), which were synthesized by a commercial company. (TaqMan SNP Genotyping Assays, Applied Biosystems, Foster City, CA.) The real-time PCR amplifications were carried out in a

final volume of 10 μl consisting of a master mix (2×) (TaqMan GTXpress Master Mix, Applied Biosystems, Foster City, CA.), a genotyping assay mix (80×) (TaqMan SNP Genotyping Assays, Applied Biosystems, Foster City, CA.) including specific primers and TaqMan MGB probes, nuclease-free water, and 2 μl of the DNA-containing solution as a DNA template. The holding stage before PCR was performed at 25ºC for 30 sec. The cycling conditions were 20 sec at 95ºC followed by 50 cycles of 3 sec at 95ºC and 20 sec at 60ºC. The holding stage after PCR was performed at 25ºC for 30 sec. In addition, an allelic discrimination plot was constructed based on the 3 types of amplification plots. Those data were calculated using software (StepOne software (version 2.1), Applied Biosystems, Foster City, CA.) based on the results obtained using DNA samples from the 13 dogs described above (i.e., 4 *MDR1* (+/+), 3 *MDR1* (+/–), and 6 *MDR1* (–/–) dogs).

Genotyping survey

The genotyping survey was carried out using DNA samples from saliva- or whole blood–spotted FTA cards of 469 Border Collies in Japan. These samples were randomly collected with the owners' informed consent for scientific evaluation of their dogs' DNA. Sample collection was performed between 2006 and the present by the Japan Border Collie Health Network (JBCHN), a volunteer breeders association for the healthy breeding of Border Collies. Genotyping was carried out using the real-time PCR assay. Direct DNA sequence analysis was carried out to confirm the sequence of *MDR1* (+/–) dogs that were identified by this genotype survey. Furthermore, the genetic relationships of these carrier dogs and their

related dogs were analyzed using the pedigree papers issued in the Japan Kennel Club and the Kennel Club of Japan.

4.1.4. RESULTS

LP analysis

On LP analysis, a 60-bp band from the wild-type allele and a 56-bp band from the mutant allele are expected to be amplified and electrophoresed at different sites on the gels reflecting different band sizes. As shown in Figure 1, the 4-bp difference was recognized on PAGE (Fig. 15A), but indistinct on agarose gel, showing 1 broad band on *MDR1* (+/–) genotype (Fig. 15B). On microchip electrophoresis, 2 amplification products were clearly separated in the gel image (Fig. 15C), allowing easy discrimination of the 3 genotypes.

MS-PCR assay

As expected, on MS-PCR assay, a 140-bp band including a longer MS-W-F primer was amplified from a wild-type allele, and a 107-bp fragment including a short MS-M-F primer was amplified from a mutant allele (Fig. 16). In the carrier dog with *MDR1* (+/–) genotype, these 2 bands of similar intensity were clearly seen on both agarose gel and microchip electrophoreses.

Real-time PCR assay

Amplification of wild-type allele tended to be slightly higher than that of mutant allele

(Fig. 17). A slight, nonspecific amplification, like wild-type allele, was observed in the *MDR1* (–/–) genotype, but the amplification did not interfere with the genotype discrimination. Consequently, the real-time PCR assay with TaqMan MGB probes clearly determined all genotypes of IT. The total required time for 50-cycle amplification was approximately 40 min. Fifty cycles of amplification were sufficient for clear genotyping. In addition, an allelic discrimination plot was constructed based on the 3 genotypes of amplification plots obtained using DNA samples from 13 dogs including 4 *MDR1* (+/+), 3 *MDR1* (+/–), and 6 *MDR1* (–/–) genotypes (Fig. 18). Three genotypes of the 13 dogs were clearly determined by this allelic discrimination plot, and the results were completely consistent with those of the direct DNA sequencing and other PCR-based assays developed in the present study.

Allele frequency and pedigree analysis

In the genotyping survey carried out on 469 Border Collies, there were no *MDR1* (–/–), 2 *MDR1* (+/–), and 467 *MDR1* (+/+) dogs, indicating that the carrier and mutant allele frequencies were 0.4% and 0.2%, respectively. Direct DNA sequencing analysis confirmed that the 2 *MDR1* (+/–) dogs detected in the current survey were heterozygous carriers for the 4-bp deletion mutation. Pedigree analysis carried out using the pedigree papers of these carrier dogs and their related dogs demonstrated that these 2 carriers had a common sire (Fig. 19).

4.1.5. DISCUSSION

Length polymorphism analysis is relatively simple because it does not require an endonuclease digestion step. However, a 4-bp difference lies on the dividing line in the separation using gel electrophoresis. In the present study, the 4-bp difference of the amplified bands was detected in the LP analysis using PAGE in combination with silver staining (Fig. 15A), but not detected in agarose gel electrophoresis in combination with ethidium bromide staining (Fig. 15B). Microchip electrophoresis separated most clearly and rapidly these 2 bands resulting in the clear discrimination of the 3 genotypes (Fig. 15C). Microchip electrophoresis has recently attracted considerable attention in DNA and RNA analysis due to its high efficiency, high throughput, timesaving ability, easy operation, and low consumption of samples and reagents (Chang et al., 2010b; Rahman et al., 2011; Zhang et al., 2003). The use of microchip electrophoresis can markedly shorten the time for analysis of DNA fragment patterns, which is currently approximately 3 min per sample. Furthermore, gel electrophoresis requires hazardous substances (acrylamide) or mutagenic (ethidium bromide) and toxic (silver nitrate) stains as well as longer analytical time, whereas the use of toxic substances and expenses incurred for hazardous waste disposal can be avoided using microchip electrophoresis. Consequently, microchip electrophoresis had advantages in detection sensitivity, timesaving, and waste management over other electrophoresis methods.

MS-PCR can be performed more simply and inexpensively than other LP analyses and real-time PCR assay because it needs only a thermal cycler and standard electrophoresis equipment using agarose gel (Rust et al., 1993). There is a non–LP-type PCR test using allele-specific primers in combination with agarose gel electrophoresis,1 but that process

needs a 2-step PCR using 2 tubes and 2 lanes for judgment in the gel electrophoresis, whereas MS-PCR can be performed in 1 tube and 1 lane, as shown in Figure 16. MS-PCR was originally developed to be used for analysis of point mutations (Rust et al., 1993), and was utilized for a single nucleotide substitution in several disorders or polymorphisms (Rahman et al., 2011; Sunder-Plassmann et al., 2005). In the present study, however, MS-PCR was applied for detection of a 4-bp deletion mutation with a modification in the design of primers shown in Table 1. The molecular difference of amplified bands was enlarged by the length of the allele-specific primers, resulting in a lower concentration of agarose gel and the use of only 1 lane of electrophoresis for a genotype determination, compared to the LP analysis and 2-step PCR, respectively.

Recently, rapid real-time quantitative PCR approaches have been developed to detect mutations in genes causing hereditary diseases in human beings (Joncourt et al., 2004; Vrettou et al., 2004) and animals (Chang et al., 2010a, b). In IT, a real-time PCR assay using TaqMan MGB probes was reported previously (Klintzsch et al., 2010). In the present study, the sequence of probes and the hybridization site of the reverse primer were changed significantly compared with the previous method. As a result, it was thought that these modifications made the genotyping discrimination markedly clear, as shown in Figures 17 and 18, compared to that in the previous report (Klintzsch et al., 2010). Furthermore, real-time PCR assay, in combination with FTA cards for sampling, markedly shortens the time required for genotyping and simplifies the procedure (Chang et al., 2010a, b) because FTA cards reduce the steps required for DNA collection, transportation, purification, and storage, consequently reducing the cost and time required to process a DNA sample to the final step of purified

DNA ready for downstream application (Mbogori et al., 2006). Therefore, among the assays developed and modified in the present study, the realtime PCR assay in combination with FTA cards for sampling was the most suitable for a large-scale survey due to its high throughput and rapidity.

The genotyping survey demonstrated that the carrier and mutant allele frequencies were 0.4% and 0.2%, respectively, in the population of Border Collies in Japan. The pedigree analysis demonstrated that the 2 carriers found in the present survey had a common sire (Fig. 19), suggesting that the mutant allele is limited to a minority of kennels for Border Collies in Japan. There are some similar reports of large-scale surveys of the mutant allele in more than 100 Border Collies in the United States (Mealey et al., 2008b; Neff et al., 2004) and Germany (Geyer et al., 2004b; Gramer et al., 2011). Two different surveys carried out in the United States demonstrated 0.99% (Mealey et al., 2008b) and 0% (Neff et al., 2004) mutant allele frequencies, respectively. In Germany, 0.60% (Geyer et al., 2004b) and 0.85% (Gramer et al., 2011) frequencies were reported in 2 different surveys. These data together with the result of the present survey suggest that the mutant allele frequency in Border Collies is markedly low compared to that in IT–susceptible breeds such as Collie (51–59%), Australian Shepherd (17–29%), and Shetland Sheepdog (6.6–30%) (Geyer et al., 2004b; Gramer et al., 2011; Mealey et al., 2008b; Neff et al., 2004).

Although IT was often responsible for the hypersensitivity for the substrate drugs of P-gp especially in Collies (Martinez et al., 2008; Mealey et al., 2008a), it was reported that non-Collie dogs lacking this mutation were affected with subchronic neurotoxicity following administration ivermectin, milbemycin, and/or moxidectin for generalized demodicosis

(Bissonnette et al., 2009). Recently, a novel insertion mutation of the canine *MDR1*/*ABCB1* gene was identified in an ivermectinsensitive Border Collie, although the correlation between the insertion mutation and the ivermectin-sensitive phenotype in this dog was not clarified completely (Han et al., 2010). In human beings, at least 3 single nucleotide polymorphisms were found in the human *MDR1*/*ABCB1* gene (Kim et al., 2001), which may be related to abnormal sensitivity to multiple drugs. Furthermore, except for P-gp, other drug-efflux transporters such as multidrug-resistance proteins or organic anion transporting polypeptides are expressed at the blood–brain barrier and these transporters function supportively to prevent entry of xenobiotics from free access to the central nervous system (Löscher and Potschka, 2005). Based on these aspects together with the low mutant allele frequency in Border Collies, it is suggested that demonstration of only IT cannot completely ensure security from adverse effects of the substrate drugs of P-gp especially in this dog breed.

Table 7. Characteristics of primers and TaqMan probes used in the present study.

Name*	Assay*	Primer/probe	Sequence† 5'→3' (mer)	Location on ORF‡	Tm (°C)	Concentration (nM)
DS-F	Direct DNA sequencing	Forward	AGGTTGGACCAGGATGGTAATAGTA (25)	Intron 3	58.8	625
DS-R	Direct DNA sequencing	Reverse	CCAATATTAACTGTAGCTCAGACTC (25)	Intron 4	57.2	625
LP-F	LP analysis	Forward	CCTCTCATGATGCTGGT (17)	193-209	52.0	625
LP-R	LP analysis	Reverse	TGAAATTCCTGCATTTGCA (19)	234-252	49.6	625
MS-W-F	MS-PCR	Forward for wild allele	ATGGAGCTGCACTCCCTCTCATGATGCTGGTTTTTGGAAACATGACA**T**A (49)	179-227	69.0	62.5
MS-M-F	MS-PCR	Forward for mutant allele	**AC**TTTTGGAAACATGAC**G**GC (20)	208-226, 231	54.3	625
MS-R	MS-PCR	Reverse	CCTCTAAGATCAGTGCCACA (20)	Intron 4	56.3	625
RT-F	Real-time PCR	Forward	CTCCCTCTCATGATGCTGGTTTT (23)	190-212	57.1	450
RT-R	Real-time PCR	Reverse	GAAAAGTTTTGTTTCTTGAAATTCCTGCAT (30)	239-268	55.9	450
Probe-VIC-W	Real-time PCR	Probe for wild allele	ATGACAGATAGCTTTGC (17)	220-236	-	100
Probe-FAM-M	Real-time PCR	Probe for mutant allele	AAACATGACAGCTTTGC (17)	216-226, 231-236	-	100

* PCR = polymerase chain reaction; DS = direct sequence; LP = length polymorphism; MS = mutagenically separated; RT = real-time; F = forward; R = reverse; W = wild-type; M = mutant-type; VIC = 6-carboxyrhodamine; FAM = 6-carboxyfluorescein. † Underlined letters in the sequence of primers are mismatched to that of the canine *MDR1*/*ABCB1* gene. ‡ ORF: open reading frame, which is the 3843-base pair sequence. The sequence and number are based on the GenBank information (accession No. NC006596).

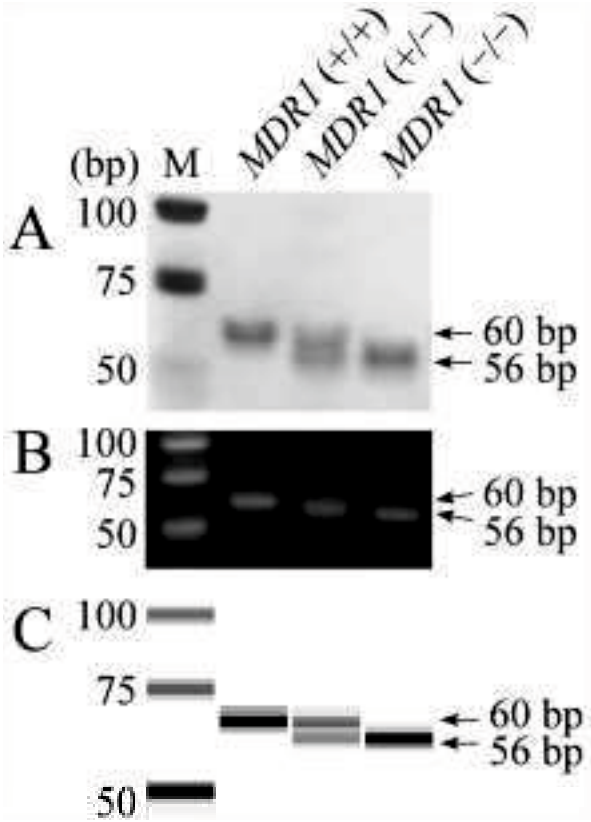

Figure 15. Electrophoretogram of polyacrylamide gel (**A**), agarose gel (**B**), and microchip (**C**) using length polymorphism analysis for a 4-bp deletion in the canine *MDR1*/*ABCB1* gene associated with ivermectin toxicosis. Fragment patterns in the 3 genotypes such as *MDR1* (+/+), *MDR1* (+/–), and *MDR1* (–/–) are shown with molecular size markers (M).

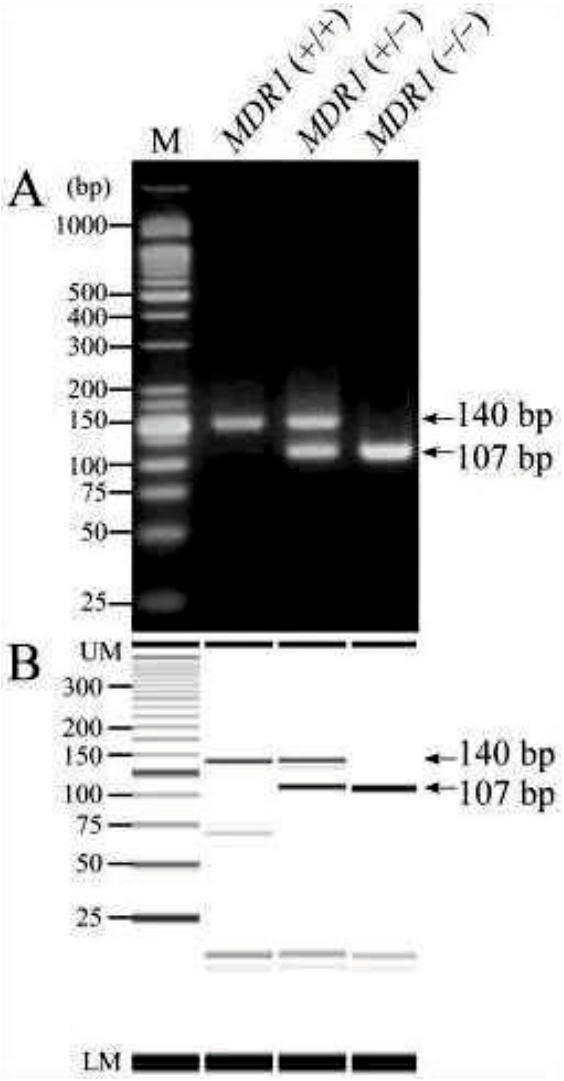

Figure 16. Electrophoretogram of agarose gel (A) and microchip (B) using mutagenically separated-PCR assay for a 4-bp deletion in the canine *MDR1*/*ABCB1* gene associated with ivermectin toxicosis. Fragment patterns in the 3 genotypes such as *MDR1* (+/+), *MDR1* (+/–), and *MDR1* (–/–) are shown with molecular size markers (M). LM = lower marker; UM = upper marker.

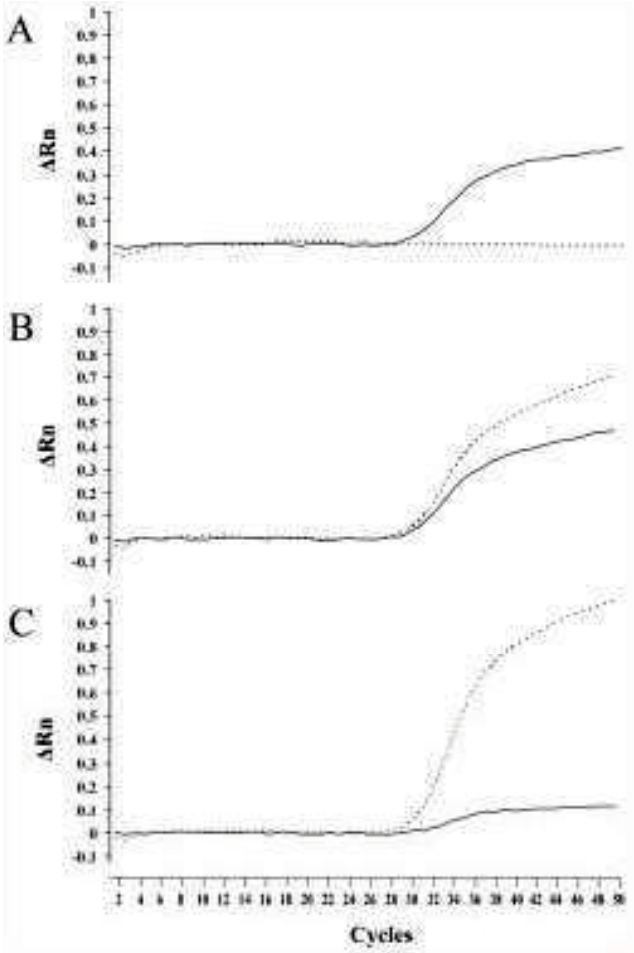

Figure 17. Real-time PCR amplification plots of wild-type and mutant alleles in a 4-bp deletion in the canine *MDR1*/*ABCB1* gene associated with ivermectin toxicosis. Amplification was plotted as fluorescence intensity (ΔRn value) against cycle number. The ΔRn value is the reporter dye signal normalized to internal reference dye and corrected for the baseline signal established in the first few cycles of reaction. Each of 3 amplification plots showed the 3 genotypes: *MDR1* (+/+; A), *MDR1* (+/–; B), and *MDR1* (–/–; C). Solid and dotted lines indicate wild-type and mutant alleles, respectively.

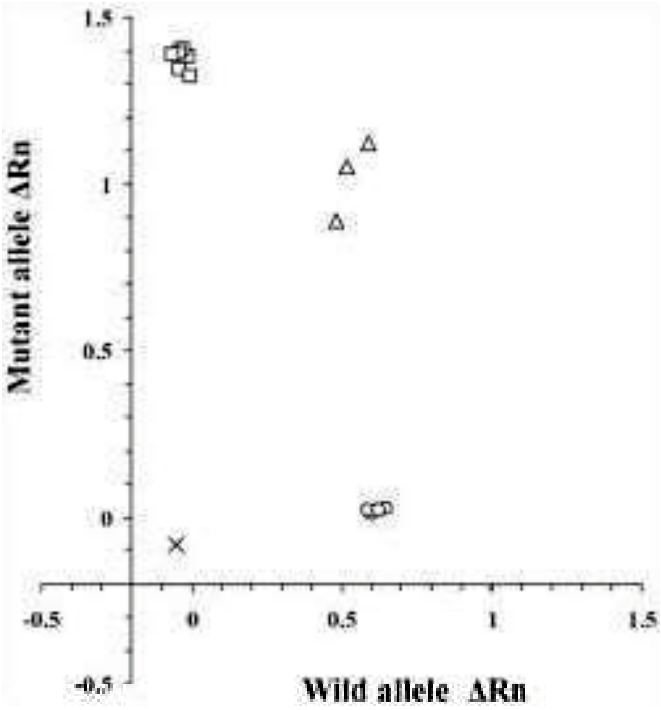

Figure 18. Allelic discrimination plot of end point fluorescence real-time PCR data showing the 3 genotypes of a 4-bp deletion in the canine *MDR1*/*ABCB1* gene associated with ivermectin toxicosis. Allelic discrimination plot was depicted using representative 13 DNA samples that had already been genotyped by direct DNA sequencing. The plot is expressed as fluorescence intensities (ΔRn value) for each allele at the X- and Y-axes. The ΔRn value in this figure is the end point reporter dye signal normalized to internal reference dye and corrected for the baseline signal established in the first few cycles of reaction. ×, no template control; ○, *MDR1* (+/+); Δ, *MDR1* (+/–); □, *MDR1* (–/–) genotypes.

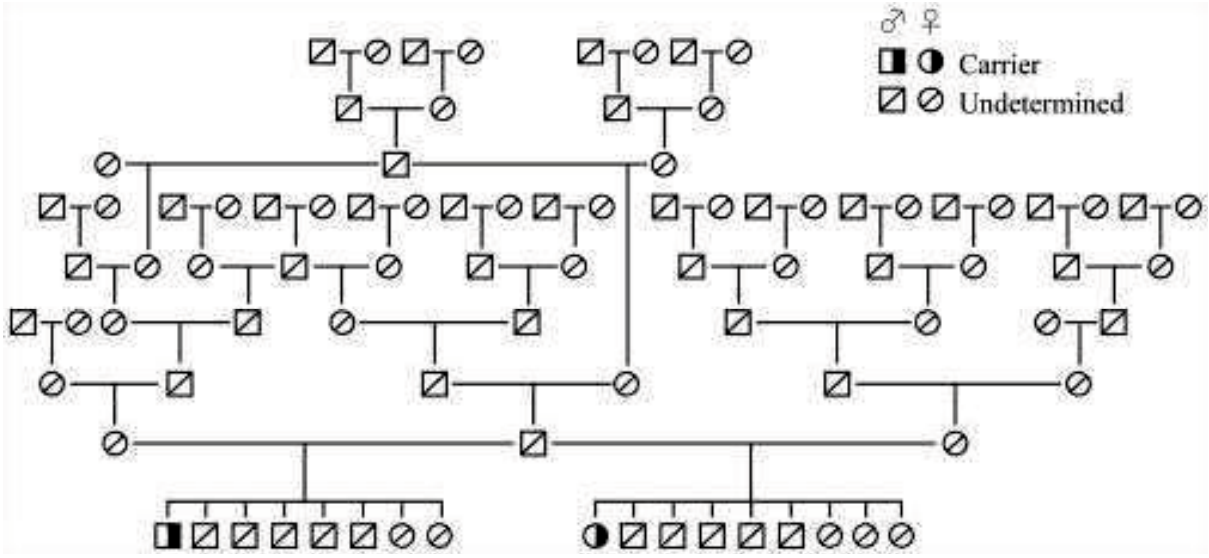

Figure 19. A genetic relationship of the 2 carrier dogs found in the genotyping survey using real-time PCR. Analysis of the 2 carriers and their related dogs was carried out using the pedigree papers issued by the Japan Kennel Club and the Kennel Club of Japan.

CHAPTER 4.2

High frequency of a single nucleotide substitution (c.-186T>G) of the canine *MDR1/ABCB1* gene associated with phenobarbital resistant idiopathic epilepsy in Border Collie dogs: a marker for therapy

The above-titled work was prepared for as: ***High frequency of a single nucleotide substitution (c.-186T>G) of the canine MDR1/ABCB1 gene associated with phenobarbital resistant idiopathic epilepsy in Border Collie dogs: a marker for therapy*** authored by:

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4.2.1. ABSTRACT

A single nucleotide substitution (c.-186T>G) associated with resistance to phenobarbital therapy has been found in the canine *MDR1*/*ABCB1* gene in Border Collies with idiopathic epilepsy. In the present study, a PCR–restriction fragment length polymorphism assay was developed for genotyping this mutation and a genotyping survey was carried out in a population of Border Collies in Japan to determine the current allele frequency. The survey demonstrated the frequencies of the T/T wild-type, T/G heterozygote, and G/G mutant homozygote to be 60.0%, 30.3%, and 9.8%, respectively indicating that the frequency of the mutant G allele is extremely high (24.9%) in Border Collies. Therefore, veterinarians should be aware of the genotypes of epileptic Border Collies before initiating drug therapy.

4.2.2. INTRODUCTION

Pharmacogenetics is a relatively new discipline that investigates how genetic variations are related to drug response and is expected to be an important tool for developing personalized medicine (Johnson, 2013). Pharmacogenetic biomarkers relevant to various diseases, drugs, and genes have been discovered and clinically used to maximize therapeutic efficacy, reduce adverse drug reactions, and to determine the most appropriate drug dosage required for efficacy and safety of treatment (Bakhouche and Slanař, 2012). Information about pharmacogenetics in domestic animals is still not as extensive as in humans. However, in veterinary medicine, there is great potential for advances in the coming years because whole genome sequencing has been completed in many domestic species (Mosher and Court, 2010).

Epilepsy is the most common neurologic disorder in dogs as well as humans (Chandler, 2006). Border Collies frequently present with severe epileptic seizures that are poorly controlled with antiepileptic drugs (Hülsmeyer et al., 2010). Recently, a single nucleotide substitution regarding phenobarbital resistant idiopathic epilepsy was found in Border Collies (Alves et al., 2011). This mutation is a substitution of thymine for guanine at intron 1 near the 5′-end of the canine *MDR1*/*ABCB1* gene (c.-186T>G), where the most important promoter elements are located (Takane et al., 2004). The mutation is not directly associated with the pathogenesis of idiopathic epilepsy, but it creates resistance to phenobarbital therapy in epileptic Border Collies (Alves et al., 2011). However, there is no data that accurately shows the frequency of this mutant allele in a normal population of Border Collies.

In the present study, a PCR–restriction fragment length polymorphism (RFLP) assay

was developed in order to discriminate the genotypes of the mutation, and a genotyping survey was carried out in Japan using samples from clinically healthy Border Collies in order to determine the frequency of the mutant allele.

4.2.3. MATERIALS AND METHODS

Control samples of each genotype, which were determined by direct DNA sequencing, were used to evaluate the accuracy of the genotyping assay. DNA templates were prepared using saliva spotted onto Flinders Technology Associates filter paper (FTA card, Whatman International Ltd., Piscataway, NJ, U.S.A.). For the PCR–RFLP assay, a 1.2-mm disc punched out of the FTA card was used as a template after quick washing as reported previously (Mizukami et al., 2012). The PCR test was carried out targeting the sequence around the mutation with forward (5′-GCA GTG GGG TGA GAA CTA GA-3′) and reverse (5′-CGC AAG CCA TGT AAG GTA TG-3′) primers in a 20-μl reaction mixture containing 10 μl of a 2× PCR master mix (GoTaq Hot Start Green Master Mix, Promega Corp., Madison, WI, U.S.A.), 12.5 pmol of primers, 1 μl of GC enhancer solution (360 GC Enhancer, Applied Biosystems, Foster City, CA, U.S.A.), and the treated disc of the FTA card as a template. After denaturation at 95°C for 10 min, 45 cycles of amplification were carried out at a denaturing temperature of 95°C for 30 sec, an annealing temperature of 60°C for 30 sec, and an extension temperature of 72°C for 30 sec. Extension during the last cycle was carried out at 72°C for 7 min and 30 sec. The PCR product was digested with a *Mbo*I restriction endonuclease (New England Biolabs Inc., Ipswich, MA, U.S.A.) at 37°C for 90 min in a 10-μl reaction mixture containing 8 μl of the PCR product, 5 U of *Mbo*I, and 1 μl of 10× restriction enzyme buffer (10× NE Buffer, New England Biolabs Inc.) included by the manufacturer. Both the unprocessed and digested PCR products were subjected to electrophoresis in 3% agarose gel (Agarose 21, Nippon Gene Co., Ltd., Tokyo, Japan). The PCR–RFLP assay was designed to digest the wild-type sequence [↓GATC] into 3 fragments and not to digest the

mutant sequence [GAGC] into 2 fragments.

The genotyping survey was carried out by using DNA templates extracted from saliva samples of 472 Border Collies aged 2 months to 14 years in Japan. These samples were collected between 2006 and 2013 by the Japan Border Collie Health Network, a volunteer breeders' association with the owners' informed consent. The PCR–RFLP assay established in this study was used for genotyping.

4.2.4. RESULTS

As shown in Table 1, a 416-base pair (bp) DNA band was amplified, in theory, in all the genotypes (Fig. 20). In the homozygous mutant-type (G/G) dog, the amplified band was digested into 2 fragments (i.e., 313- and 122-bp bands) because there was only 1 restriction site available to the *Mbo*I, which was present in all the genotypes and unrelated to the target sequence. In the homozygous wild-type (T/T) dog, the amplified band was digested into 3 fragments (i.e., 191-, 122-, and 103-bp bands) because there were 2 restriction sites available: one at the target sequence [↓GATC] and another at the same sequence present in all the genotypes. The amplification product in the heterozygous (T/G) dog was digested into 4 fragments (i.e., 313-, 191-, 122-, and 103-bp bands). Owing to the inseparability of the 122- and 103-bp bands, these 2 bands externally appeared to be 1 band; therefore, the digested PCR products in the T/T wild-type and T/G heterozygous dogs externally appeared as 2 and 3 fragments, respectively. The genotypes of all of the dogs examined were consistent with the results of direct DNA sequencing.

In the survey on 472 Border Collies, 283 (60.0%) were T/T wild-type dogs, 143 (30.3%) were T/G heterozygous dogs, and 46 (9.8%) were G/G homozygous mutant-type dogs. The overall frequency of the mutant G allele is 24.9%.

4.2.5. DISCUSSION

In the PCR–RFLP assay, all the genotypes could be easily discriminated by confirming the presence and/or absence of 313- and 191-bp fragments via agarose gel electrophoresis (Fig. 20). The restriction site that is present in both alleles, and unrelated to the target sequence, did not interfere with the determination of all the genotypes but provided assurance of the digestive function of the restriction endonuclease. Furthermore, other genotyping assays (including a TaqMan probe-based real-time PCR method) could not be established because nucleic acid sequences around the mutation are rich in GC pairs and include a partially-duplicated repeat (GenBank reference no. NC_006596 and accession no. AAEX03009295.1), which might interfere with specific hybridization by designed probes. Therefore, the PCR–RFLP assay developed in this study is a very useful tool for the genotyping of c.-186T>G.

The genotyping survey showed that the mutant G allele frequency in Border Collies is extremely high (approximately 25%) compared to that in other molecularly-defined, fetal inherited diseases in Border Collies, such as neuronal ceroid lipofuscinosis (4.1%) and trapped neutrophil syndrome (5.6%) (Mizukami et al., 2013). The phenotype of the mutant G allele seems to act in a dominant fashion (Alves et al., 2011): as a consequence, approximately 40% of Border Collies (G/G and T/G genotypes) examined in this study may show resistance to phenobarbital therapy when they become affected with epilepsy. This may be the reason why drug-resistant epilepsy frequently occurs in Border Collies (Hülsmeyer et al., 2010). Further studies are needed to clarify this issue. However, the mutation is not directly pathogenic (Alves et al., 2011) and there are alternative therapeutic options to

phenobarbital for canine epilepsy (von Klopmann et al., 2007). Therefore, owing to the increased risk of an elevation of inbreeding coefficient, it is not necessarily appropriate to aggressively remove the mutant G allele from a population of Border Collies. Instead, when epileptic Border Collies are treated with antiepileptic drugs, the genotypes should be determined in advance in order to choose the appropriate pharmacological therapy.

Previous studies hypothesized that phenobarbital resistance is due to an up-regulation of the *MDR1*/*ABCB1* gene and the subsequent overexpression of this gene's product, P-glycoprotein (P-gp), in the brain of Border Collies with drug-resistant epilepsy (Alves et al., 2011). P-gp is expressed at the luminal membrane of endothelial cells of the brain capillaries and restricts the entry of drugs (including ivermectin and phenobarbital) into the central nervous system by an efflux-based transport mechanism (Mizukami et al., 2012; Schinkel, 1997). P-gp is also expressed in various mammalian tissues such as the brush border membrane of epithelial cells in the intestinal tract, the luminal membrane of proximal tubules in the kidney, and the canalicular membrane of liver hepatocytes (Thiebaut et al., 1987). Over-expression of P-gp in these organs diminishes oral drug bioavailability and promotes drug elimination into urine and bile (Fromm, 2000). Therefore, c.-186T>G in Border Collies may not only affect phenobarbital resistance but may also affect the pharmacokinetics of other P-gp substrate drugs. Additionally, in veterinary medicine, there is another well-known mutation in the canine *MDR1*/*ABCB1* gene (c.227_230del) which causes a P-gp defect and, subsequently, substrate drug intoxication, including ivermectin toxicosis (Mealey et al., 2001); however, although the allele frequency of c.227_230del is low (0.25%) in Border Collies (Mizukami et al., 2012b). Veterinarians should be aware of these genetic markers of

the canine *MDR1*/*ABCB1* gene that influence therapeutic efficacy and toxicity thresholds.

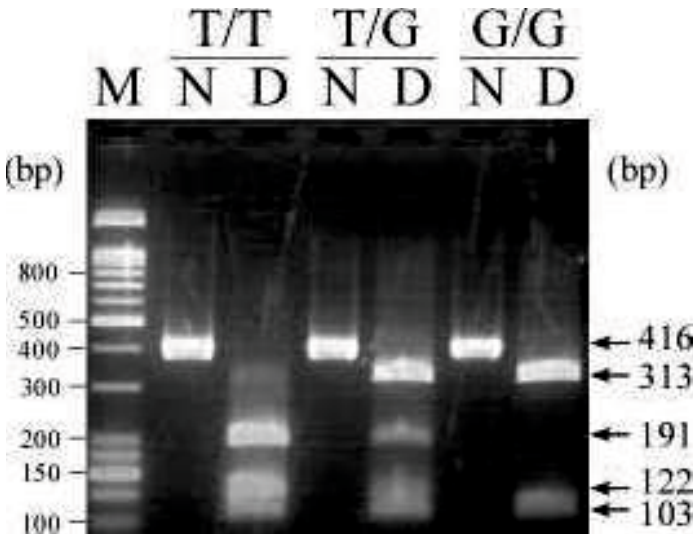

Figure 20. Electrophoretogram of agarose gel using PCR–restriction fragment length polymorphism assay. Fragment patterns in the 3 genotypes such as homozygous wild-type (T/T), heterozygote (T/G), and homozygous mutant-type (G/G) are shown with molecular size markers (M). The amplified DNA without digestion (N) and DNA digested with restriction endonuclease *Mbo*I (D) were analyzed simultaneously by electrophoresis. bp = base pairs.

CHAPTER 4.3

Case study and molecular epidemiology of collie eye anomaly in Hokkaido dogs

The above-titled work originally appeared in "*Veterinary Ophthalmology* (Mizukami et al., 2012)" as: ***Collie eye anomaly in Hokkaido dogs: case study*** authored by:

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4.3.1. ABSTRACT

This study was performed to describe a Hokkaido dog, one of the traditional Japanese breeds that was affected by Collie eye anomaly (CEA), and to report the genotype of this dog and the Hokkaido dog allelic frequency of the CEA-associated mutation. A nine-month-old intact female Hokkaido dog without any obvious visual disturbance was diagnosed ophthalmoscopically with CEA. Severe choroidal hypoplasia was observed in the bilateral temporal area adjacent to the optic nerve head, appearing as whitish areas. Therefore, the dog was suspected of possessing the CEA-associated mutation that was previously reported as an intronic 7.8-kilo base deletion in the canine *NHEJ1* gene. SYBR Green-based real-time PCR with a melting curve analysis, conventional PCR with agarose gel electrophoresis, and direct DNA sequencing were carried out to determine the genotype of the dog. Furthermore, a preliminary genotyping survey was carried out in 17 Hokkaido dogs from three kennels using the real-time PCR method, and the pedigree relationships were analyzed using their pedigree papers. The Hokkaido dog affected by CEA was proven to possess the CEA-associated mutation. Of these 17 Hokkaido dogs, 12 dogs were heterozygous carriers and five dogs were affected by this mutation. The preliminary genotyping survey and pedigree analysis demonstrated that the allelic frequency of the CEA-associated mutation is very high in Hokkaido dogs. These data suggest that the Hokkaido breed is highly susceptible to CEA because of the known CEA-associated mutation much like the Collie-related breeds.

4.3.2. INTRODUCTION

Collie eye anomaly (CEA) is a congenital inherited canine ocular disorder affecting the posterior segment of the eye (Bedford et al., 1982b, 1998; Roberts, 1969; Bjerka°s, 1991). CEA is a pleomorphic syndrome, with variability in manifestation and severity of clinical and ophthalmologic lesions. The two main ophthalmoscopic changes are regional choroidal hypoplasia and coloboma of the optic disk or adjacent areas, which may be bilateral and are often symmetrical with equal severity. Clinically, the onset of CEA results in an ophthalmoscopically detectable window defect in the ocular fundus located temporal to the optic nerve (Lowe et al., 2003). Defects of the sclera characterized by colobomatous lesions may also occur presenting as pits within or engulfing the optic nerve head or in the adjacent fundus. Mildly to moderately affected individuals appear to retain normal visual function throughout life as determined by behavioral observation and clinical electroretinography. However, severely affected dogs, particularly those with colobomas, can develop retinal detachment and intraocular hemorrhage leading to blindness, although bilateral blindness rarely occurs.

Many studies have been performed to determine the mode of inheritance for this syndrome (Roberts, 1969; Donovan, 1965, 1969; Wyman and Donovan, 1969; Yakely et al., 1968, 1972; Wallin-Håkanson et al., 2000). Variability in the severity and expression of the disorder is well recognized in the CEA phenotype and suggests that CEA may be a complex trait with multiple genetic contributors (Lowe et al., 2003). However, recently, a mutation was demonstrated by fine-mapping study across multiple dog breeds as an intronic deletion of 7799 base pairs (bp) in the *NHEJ1* gene, enabling detection of both homozygotes and

heterozygotes by a conventional polymerase chain reaction (PCR)–based diagnostic test (Parker et al., 2007). Furthermore, a rapid DNA assay using SYBR Green-based real-time PCR was also developed to detect this mutation (Chang et al., 2010b).

Collie eye anomaly has historically been clinically characterized as a hereditary disorder segregated in Collie-related breeds including Australian Shepherd, Border Collie, Lancashire Heeler, Rough Collie, Shetland Sheepdog, and Smooth Collie (Bedford, 1982a 1982b, 1998; Rubin et al., 1991). Molecularly defined CEA has recently been recognized in 11 breeds: Australian Shepherd; Australian Shepherd (Miniature); Border Collie; Boykin Spaniel; Collie (Rough); Collie (Smooth); Lancashire Heeler; Longhaired Whippet; Nova Scotia Duck Tolling Retriever; Shetland Sheepdog; and Silken Windhound (Parker et al., 2007; http://www.optigen.com). Notably, all of these are Collie-related breeds. Lesions that resemble CEA clinically are also observed in some dogs as a variable expression of the Merle phenotype – e.g., Catahoula Leopard Dog, Dachshund (all varieties), and Great Dane (Parker et al., 2007) – but this phenotype is molecularly distinct from CEA (Clark et al., 2006). Clinical phenotypes resembling CEA have also been observed occasionally in various other non-Collie breeds including Beagle, German Shepherd, Miniature or Toy Poodle, and mixed-breed dogs (Parker et al., 2007; Priester, 1972; Rampazzo et al., 2005; American College of Veterinary Ophthalmologists, 2007), but the molecular causes of these phenotypes have not been determined.

This case study is the first report of CEA observed in one of the traditional Japanese breeds, Hokkaido dog. The affected dog was diagnosed by ophthalmoscopy and molecular methods. In addition, pedigree analysis was carried out to determine the allelic frequency in

the population of Hokkaido dogs.

4.3.3. MATERIALS AND METHODS

Case

A nine-month-old intact female Hokkaido dog with a white coat was referred for entropion of the lower eyelid with associated corneal pigmentation. There were no visual deficits observed. A routine ophthalmoscopic examination revealed severe choroidal hypoplasia bilaterally and temporal to the optic nerves (Fig. 21). No other posterior segment abnormalities were noted. The rest of the physical and ophthalmic examination including intraocular pressure measurements and Schirmer tear tests was normal. After 17 days, the entropion was corrected surgically with the secondary corneal pigmentation improving as a consequence. Based on the ophthalmologic examination, this dog was diagnosed with CEA and suspected of having the CEA-associated mutation.

Collection of DNA and pedigree information

The affected female dog was considered for breeding in a kennel (A) specializing in Hokkaido dogs. This dog had originated from kennel B that possessed the dam. The sire was from a different kennel (C). Saliva specimens were obtained from the affected dog and seven other dogs in kennel A, from nine dogs including the dam in kennel B, and from the sire in kennel C, using Flinders Technology Associates filter paper (FTA card; Indicating FTA Classic Card; Whatman International Ltd., Piscataway, NJ, USA) and FTA special applicator

to swab saliva (Sterile Foam Tipped Applicator; Whatman International Ltd.). As a positive control specimen, saliva from a male Rough Collie that was known to have a heterozygous carrier genotype was obtained from a kennel specializing in Collie breeds. Saliva-spotted FTA cards and pedigree papers were sent directly from the kennels to our laboratory after obtaining informed consent of the breeders. DNA on FTA cards was used for all genetic analyses in this study. Pedigree analysis was carried out using pedigree papers issued from three different kennel clubs specializing in Hokkaido dogs (http://www.doukenkyou.com; http://hokkaidoinu.jp).

Genetic tests

The genotype of the CEA-associated mutation in the affected dog was determined using three types of genetic tests, i.e., SYBR Green-based real-time PCR with a melting curve analysis (Chang et al., 2010b), conventional PCR with agarose gel electrophoresis(Parker et al., 2007; Chang et al., 2010b), and direct DNA sequencing. Real-time PCR and conventional PCR were carried out according to the protocols and conditions reported previously (Chang et al., 2010b). Direct DNA sequencing was carried out to confirm the DNA sequence around the 7799-bp deletion. PCR was performed using forward (F20: 5'-TGGGCTGGTGAACAT TTGTA-3') and reverse (RM: 5'-ACCAATCATCCAGCCCAGCATTTAA-3') primers. The target PCR product (279 bp) was separated on agarose gel and excised and purified using the gel extraction kit (QIAquick Gel Extraction Kit; QIAGEN, Tokyo, Japan). Direct cycle sequencing of the purified PCR product was carried out by a commercial company (Hokkaido

System Science Co. Ltd., Sapporo, Japan). The genotypes of all other dogs were determined using the real-time PCR method.

4.3.4. RESULTS

The result of the SYBR Green-based real-time PCR with a melting curve analysis on the affected dog is shown in Fig. 22. Only the mutant allele was amplified, suggesting a mutant homozygote. In the melting curve analysis after PCR amplification, a single peak corresponding to the mutant product in temperature was obtained, suggesting the specific amplification without any nonspecific reactions. In the conventional PCR, only mutant product of 941-bp in length was recognized on agarose gel electrophoresis (Fig. 23), also suggesting the mutant homozygote. Furthermore, the result of the direct DNA sequence demonstrated that the genome of the affected dog lacked the 7799-bp region in intron 4 of the canine *NHEJ1* gene (Fig. 24).

Of the 17 Hokkaido dogs examined, 12 dogs including the parents of the affected dog were heterozygous carriers and the remaining five dogs were mutant homozygotes, i.e., CEA-affected dogs. Regarding this mutation, there were no normal or clear dogs. The pedigree analysis demonstrated that 13 dogs had a genetic relationship with the affected dog as shown in pedigree 1 in Fig. 25. However, the other four dogs did not have a genetic relationship with the affected dog, although they were connected with each other as shown in pedigree 2 in Fig. 25.

4.3.5. DISCUSSION

This is the first reported occurrence of CEA in the Hokkaido dog breed. The diagnosis of CEA in the affected dog was established ophthalmologically and genetically based on the CEA-specific findings of ophthalmoscopy and the results of the genetic tests. The result of direct DNA sequencing demonstrated that the mutation of the affected dog was the same as the CEA-associated mutation reported previously in Collies and other Collie-related breeds (Parker et al., 2007).

It is commonly thought that dogs affected with CEA belong to one of the herding breeds with Collie ancestry (Parker et al., 2007). In the previous surveys, the prevalence of CEA has been estimated to be 70–97% for Rough and Smooth Collies in the United States and Great Britain (Bedford et al., 1982; Yakely, 1972), 68% for Rough Collies in Sweden (Wallin-Håkanson et al., 2000), 72% for Shetland Sheepdogs in Great Britain (Bedford et al., 1982b), 13.7% for Lancashire Heelers in Great Britain (Bedford et al., 1998), 6% for Border Collies (Bedford et al., 1982a), and 4% for Australian Shepherds in Australia (Munyard et al., 2007). At present, these six Collie-related breeds are recognized to be susceptible to CEA.

In the present study, a preliminary genotyping survey was carried out on 17 Hokkaido dogs from three different kennels and demonstrated that they all had at least one mutant allele, i.e., five affected dogs, 12 carriers, and no normal dogs. Furthermore, pedigree analysis suggested that affected and carrier dogs seemed to be distributed widely in the population of Hokkaido dogs, not limited to the close family related to the affected dog. The correlation between phenotype and genotype was not determined in Hokkaido dogs because none of the dogs except the affected dog underwent ophthalmoscopy to detect CEA-related findings.

However, the data obtained in this study suggest that the Hokkaido breed is highly susceptible to CEA because of the known CEA-associated mutation much like those six Collie-related breeds with a predilection for CEA.

There is a question about how the CEA-associated allele became segregated in Hokkaido dogs. The possible reasons include: (i) there may have been an unsuspected historical admixture of Collie-related dogs into the Hokkaido breed, (ii) the CEA-associated mutation in Hokkaido dogs might be an independent deletion, and (iii) the CEA-associated mutation may have originated as a much more ancient event than previously suspected and have entered the Hokkaido and Collie-related gene pools from a very ancient common ancestor. The first speculation would be ruled out because of the large difference in appearance between Hokkaido and Collie-related dogs. An accidental but exactly identical deletion in the second speculation is unlikely to occur stochastically. Therefore, the last speculation is the most likely. Several reports of CEA-like disorder in non-Collie breeds might support this theory, although the findings have not yet been molecularly defined (Parker et al., 2007; Priester, 1972; Rampazzo et al., 2005; American College of Veterinary Ophthalmologists, 2007). This question could be resolved by careful SNP genotyping, haplotype, and linkage disequilibrium analyses.

The Hokkaido dog is one of the traditional Japanese breeds and was originally used for hunting, but is now mainly bred for show or as guard dogs, and is a protected species (http://www.doukenkyou.com; http://hokkaidoinu.jp). Compared to other Japanese breeds, such as the Akita and Shiba Inu, the population of Hokkaido dogs is limited. Among these breeds, the Akita was described as one of the breeds with sporadic isolated colobomas in the

supplementary data of the report on CEA-associated mutation (Parker et al., 2007), but this description of the Akita was not supported by genetic verification. Further work on these Japanese dog breeds is needed to elucidate the prevalence of CEA and CEA-associated mutation and to eradicate this unfavorable allele.

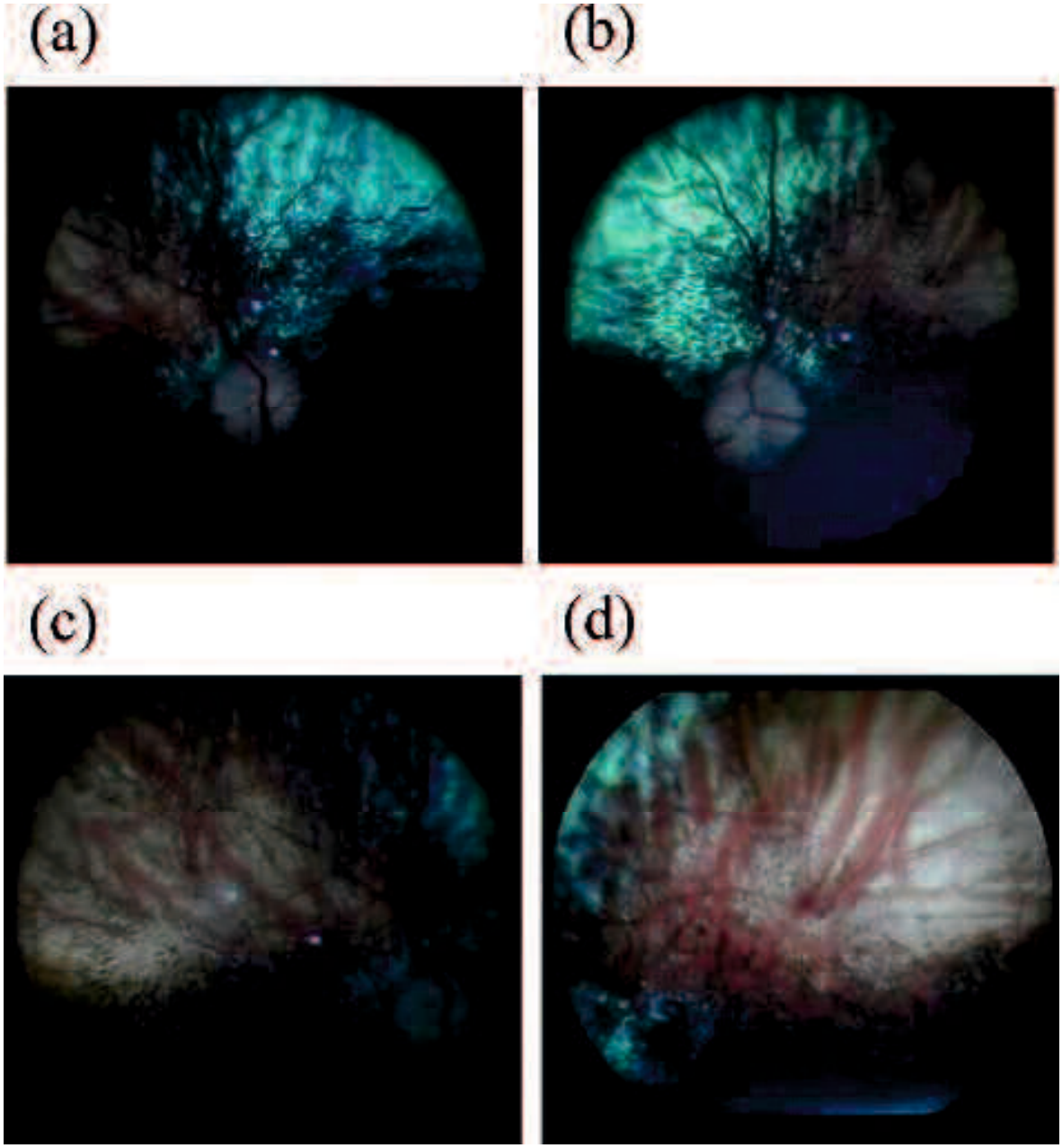

Figure 21. Fundus photographs of right (a and c) and left (b and d) eyes in the affected Hokkaido dog. In both fundi, a whitish zone of extensive choroidal hypoplasia is located in the temporal area with the absence of tapetum lucidum (a and b). The sclera and choroidal vessels can be seen in the region of choroidal hypoplasia (c and d).

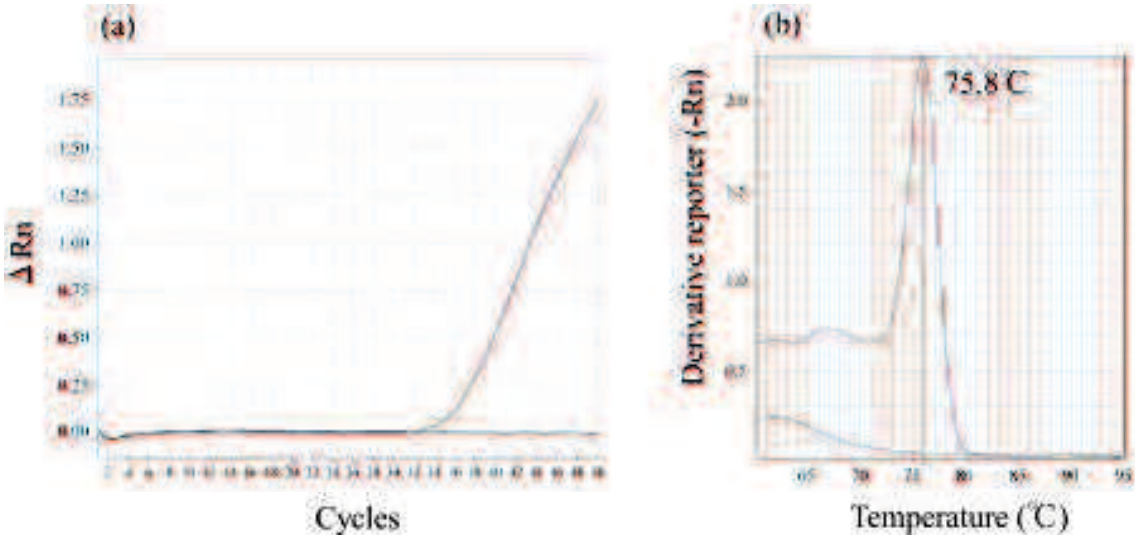

Figure 22. Collie eye anomaly genotyping by SYBR Green-based real-time PCR assay with a melting curve analysis. (a) Real-time PCR amplifications of wild-type (blue line) and mutant (red line) alleles were carried out in two separate tubes in the same PCR run and presented in an overlapped plot. This amplification plot indicated that only amplification reaction using primers for mutant allele proceeded. (b) Melting curve analysis was carried out after the real-time PCR assay. Red and blue lines indicate melting curves of wild-type and mutant alleles, respectively. The melting temperature of the PCR product using primers for mutant allele corresponded to that of mutant product reported previously. The blue flat curve on the lower side was observed in the tube for wild-type allele.

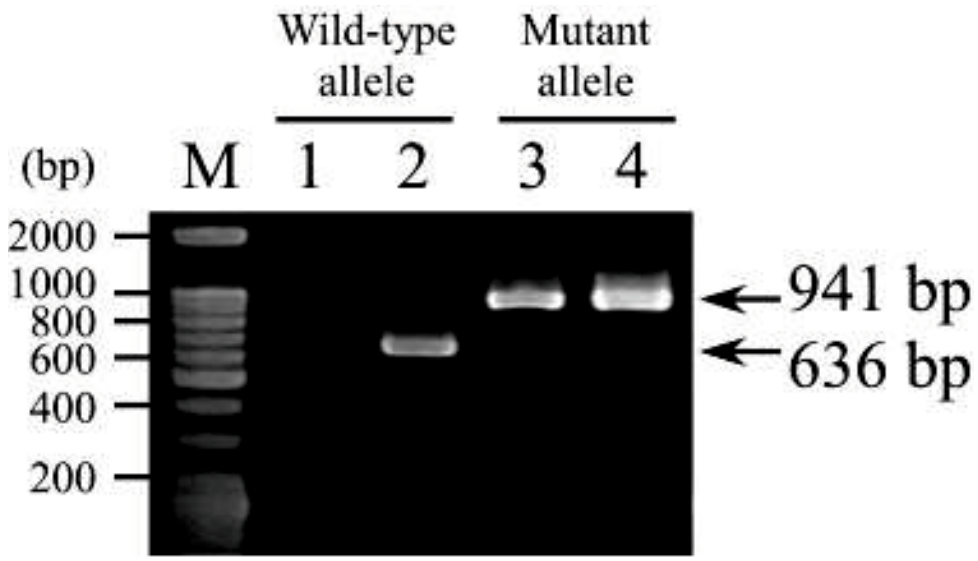

Figure 23. Agarose gel electrophoresis after a conventional PCR assay for collie eye anomaly. Lane M shows molecular size marker. Lanes 1 and 2 show the existence of wild-type allele product, while lanes 3 and 4 show that of mutant allele product. Lanes 1 and 3 are PCR products in the affected dog, and lanes 2 and 4 are those in a Rough Collie with a heterozygous carrier genotype.

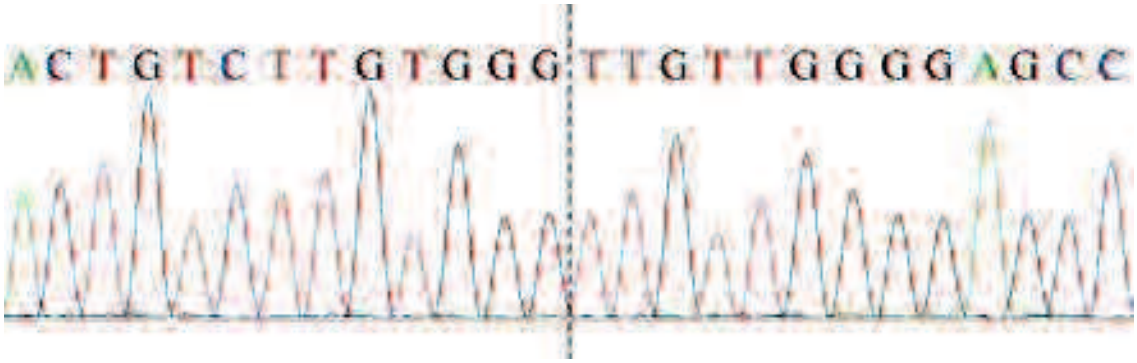

Figure 24. An electropherogram of the canine *NHEJ1* gene from the affected Hokkaido dog. The dog chromatograph shows intronic deletion of 7799 bases in the canine *NHEJ1* gene (dotted line).

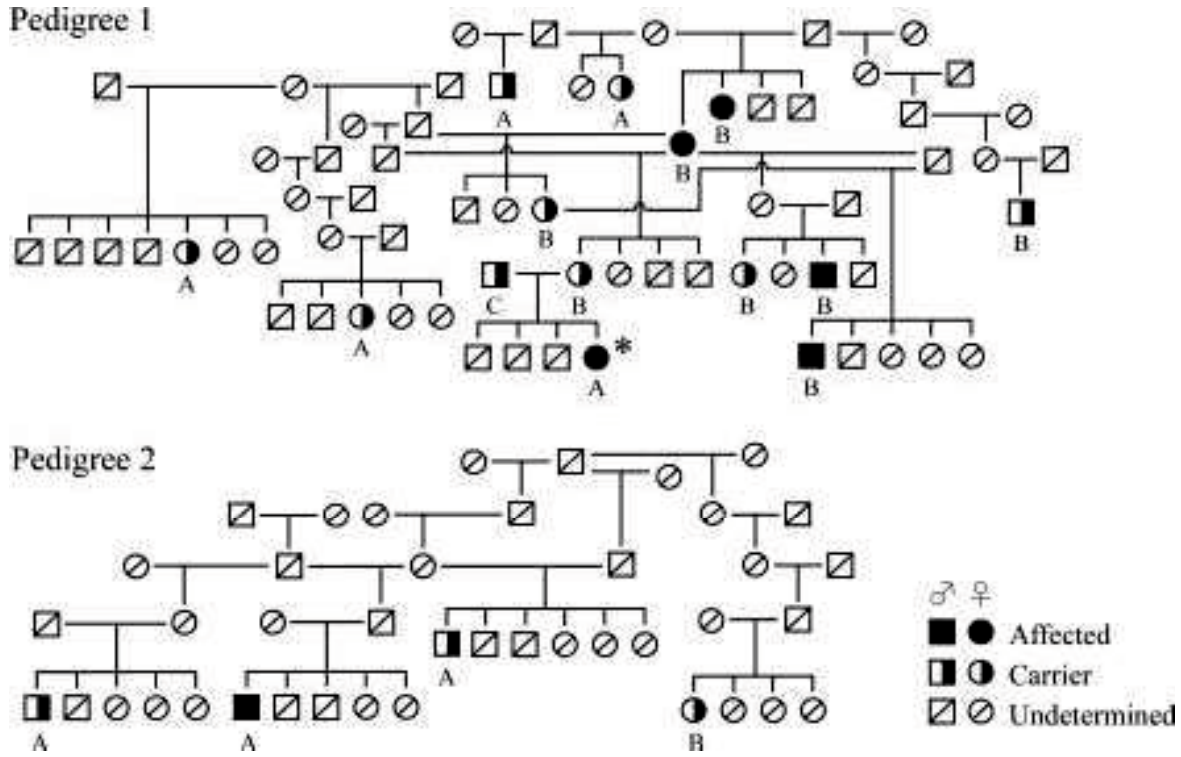

Figure 25. A genetic relationship of Hokkaido dogs examined CEA genotypes. Genotypes were determined using SYBR Green-based real-time PCR assay with a melting curve analysis. *: Affected dog; A, B, and C: kennels.

CHAPTER 4.4

Analysis of genetic relationship between the mutations associated with Collie eye anomaly and ivermectin toxicosis in various dog breeds

The above-titled work was prepared for as: ***Analysis of genetic relationship between the mutations associated with Collie eye anomaly and ivermectin toxicosis in various dog breeds*** authored by:

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4.4.1. ABSTRACT

The mutation associated with Collie eye anomaly (CEA) and a 4-bp deletion in the canine *MDR1*/*ABCB1* gene are common in Collie-related breeds, suggesting a strong genetic relationship between the 2 defects. However, the Hokkaido Inu, a Japanese breed unrelated to Collies, has unusually high frequency of the CEA mutation. The aim of this study was to investigate the genetic relationship between the 2 defects. Nine breeds including Collie-related and Japanese breeds were evaluated using real-time PCR methods. Collies had very high frequencies of both defects while Border Collies had high CEA but low MDR1 gene defect frequencies. Hokkaido Inus had only the CEA, whereas Ryukyu Inus had only the MDR1, gene defect. These data suggest that there is no relationship between the 2 defects.

4.4.2. INTRODUCTION

Collie eye anomaly (CEA) is an inherited canine ocular disorder causing regional choroidal hypoplasia and coloboma of the optic disk or adjacent areas, and is associated with a 7.8-kilo base pair (bp) deletion in the canine *NHEJ1* gene (Parker et al., 2007). A 4-bp deletion in the canine *MDR1*/*ABCB1* gene (*MDR1* gene defect) causes neurotoxicosis after administration of P-glycoprotein substrates such as ivermectin (Gramer et al., 2011). These 2 mutations are common in Collie-related and sighthound breeds, indicating a strong genetic relationship between the 2 defects, although these canine genes are located on different chromosomes: *NHEJ1* on chromosome 37 and *MDR1* on chromosome 14 (Gramer et al., 2011; Parker et al., 2007).

Recently, a very high frequency of the CEA mutation has been reported in the Hokkaido Inu, a traditional Japanese breeds; this is the first identification of a CEA-predisposed breed unrelated to Collies or sighthounds (Mizukami et al., 2012a). However, prevalence of the *MDR1* gene defect in the Hokkaido Inu is unknown. Furthermore, the genetic relationship between the CEA and *MDR1* mutations in various dog breeds including Japanese breeds has not been determined. This study investigated the genetic relationship between these 2 mutations in multiple dog breeds.

4.4.3. MATERIALS AND METHODS

In this study, 1,110 DNA samples were collected from 9 dog breeds including 2 Collie-related breeds (Collie and Border Collie), 1 sighthound (Saluki), 2 non-Collie European breeds (Toy Poodle and Miniature Dachshund), 3 Japanese breeds (Hokkaido, Shiba, and Ryukyu Inu), and the Korean Jindo. These breeds were chosen for the following reasons. Collie-related breeds are known to commonly carry both mutations (Gramer et al., 2011; Parker et al., 2007). The Saluki is a sighthound that has not been demonstrated to carry the mutations. The Toy Poodle and Miniature Dachshund possibly carry the CEA mutation because sporadic cases of CEA-like ophthalmological signs have occurred in these breeds (Parker et al., 2007). Japanese breeds are suspected to carry the mutations because the Hokkaido Inu has very high frequency of the CEA mutation (Mizukami et al., 2012a). The Korean Jindo is also suspected to carry the mutations because this breed has similar appearance and inherited traits to the Japanese breeds (Yamato et al., 1999). The number of dogs in each breed is shown in Table 1. The genotypes of the 2 mutations were determined using real-time PCR methods reported previously (Chang et al., 2010; Mizukami et al., 2012b).

4.4.4. RESULTS

The results of this study are shown in Table 8. Collies had very high frequency of both mutations. Border Collies had high frequency of the CEA mutation, but very low frequency of the *MDR1* gene defect. Hokkaido Inus did not show the *MDR1* gene defect but displayed very high frequency of the CEA mutation. Ryukyu Inus did not show the CEA mutation, but 3 of 20 dogs carried the *MDR1* gene defect. The other breeds did not carry either mutation.

4.4.5. DISCUSSION

The results of the present study demonstrated that the CEA and *MDR1* gene defects do not always coexist in canine breeds even if they are predisposed toward one of these defects. This suggests that there is not a complete genetic relationship between the 2 defects, although statistical analysis could not be performed because of the small number of breeds possessing one or both of the mutations. Collies had markedly high frequencies of both mutations as previously reported (Gramer et al., 2011; Parker et al., 2007). Border Collies also had high frequency of the CEA mutation but very low frequency of the *MDR1* gene defect, as first demonstrated in the same population previously (Mizukami et al., 2012b). The Saluki has long been considered an "ancient" breed; however, a recent genetic study demonstrated that ancient breeds do not resemble early domestic dogs more closely but have rather avoided recent admixture with other breeds, likely facilitated by geographical and cultural isolation (Larson et al., 2012). Therefore, the reason why the Saluki had neither of the mutations might be that these mutations have not been introduced because of isolation in the course of establishment of this breed. The Toy Poodle and Miniature Dachshund had neither of the mutations, although sporadic cases having ophthalmological lesions similar to those in CEA have been found in these breeds (Parker et al., 2007). These lesions are observed in non-CEA disorders including merle syndrome (Parker et al., 2007); therefore, disorders such as merle syndrome might cause similar ocular lesions in these 2 breeds.

The results demonstrated negligible relationship between the CEA and *MDR1* mutations in the Japanese and Korean breeds. Hokkaido Inus had very high frequency of the CEA mutation as previously reported (Mizukami et al., 2012a), but no *MDR1* gene defect, in

this study. However, the pattern seen in the Hokkaido Inu breed was not observed in other Japanese and Korean breeds. Neither mutation was found in the Japanese Shiba Inu and Korean Jindo dogs. However, in the Ryukyu Inu breed, 3 of 20 dogs had the *MDR1* gene defect, although none carried the CEA mutation.

The *MDR1* gene defect has also been found in Old English Sheepdogs, German Shepherds, and Wällers as well as Collie-related dogs and sighthounds (Gramer et al., 2011). There are several possible origins of the *MDR1* gene defect. All the dogs carrying this mutation are thought to be descendants of a dog that lived in Great Britain before genetic isolation of pure breeds by registered breeding (Fecht and Distl, 2008). However, the ancestors of Ryukyu and Hokkaido Inus are believed to have been brought to the Japanese archipelago from the Asian Continent by ancient people 10 to 12 thousand years ago (Tanabe, 2006). These 2 breeds have been isolated in southern (Okinawa, alias Ryukyu) and northern (Hokkaido) islands, respectively. In view of the postulated migration route and the isolated habitats of these dogs, the origin of the CEA and *MDR1* mutations might exist in more primitive dogs than once thought. Further studies are needed to verify this hypothesis.

In conclusion, no genetic relationship was found between the CEA and *MDR1* mutations in various dog breeds. These 2 mutations existed independently in breeds that are not genetically related such as Collies and Japanese breeds. A possible explanation is that these mutations originated in ancient dogs and resultant phenotypes had neutral or only weak effects on survival and selection. Veterinarians should be aware that predispositions toward these 2 defects differ considerably depending on breed.

Table 8

The number of dogs with each genotype (carrier and affected) and allele frequency for the Collie eye anomaly and *MDR1* mutations in various dog breeds.

Breed	Number	Collie eye anomaly			*MDR1* gene defect		
		Carrier	Affected	Frequency	Carrier	Affected	Frequency
Collie	9	0	9	1	3	6	0.833
Border Collie	437	128	9	0.167	2	0	0.002
Saluki	29	0	0	0	0	0	0
Toy Poodle	229	0	0	0	0	0	0
Miniature Dachshund	232	0	0	0	0	0	0
Hokkaido Inu	18	12	6	0.667	0	0	0
Ryukyu Inu	20	0	0	0	3	0	0.075
Shiba Inu	87	0	0	0	0	0	0
Jindo	49	0	0	0	0	0	0

CHAPTER 5

Molecular epidemiologic research on multiple genetic disorders in pure dog breeds: a simulation analysis of prevention

The above-titled work was prepared for as: ***Molecular epidemiologic research on multiple genetic disorders in pure dog breeds: a simulation analysis of prevention*** authored by:

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5.1. ABSTRACT

In veterinary medicine, it is necessary to prevent genetic disorders in pure dog breeds in order to maintain the quality of these companion animals. The objectives of this study are to examine the allele frequencies associated with multiple genetic disorders in the same population of a pure dog breed and to perform a simulation analysis of prevention of these genetic disorders. Border Collies and 7 known genetic disorders were chosen as the target of this study. Genotyping was performed using each PCR methods. The ratio of animals without all 7 mutant alleles was 56.1%. This finding suggested that the prevention to construct the population composed by dogs free from these 7 genetic disorders can be performed with a minimum of loss of genetic diversity.

5.2. INTRODUCTION

Canine genetic disorders play a crucial role in understanding of the molecular mechanisms and developing of novel therapies for human (Lequarré et al., 2011). In veterinary medicine, it is necessary to prevent and control genetic disorders in pure dog breeds using reproductive management in order to maintain the quality of these companion animals (Mizukami et al., 2012b). As with a human, many genes have been found in a dog since canine whole genome sequence was completed approximately 10 years ago. Over 150 disorder-causing or associated mutations have been identified in dogs and the information is currently available in the OMIA database (Online Mendelian Inheritance in Animals). This advancement in canine genetics has contributed to many preventive measures against genetic disorders in pure breed dogs. However, a comprehensive study aimed at prevention of actual multiple genetic disorders has not yet been performed in dogs. The objectives of this study are to examine the allele frequencies associated with multiple genetic disorders in the same population of a pure dog breed and to perform a simulation analysis of prevention of these genetic disorders.

5.3. MATERIALS AND METHODS

The Border Collie breed was chosen as the target for this study because there are 7 molecularly defined genetic disorders in Border Collies, i.e., neuronal ceroid lipofuscinosis (NCL, OMIA 001482-9615), trapped neutrophil syndrome (TNS, OMIA 001428-9615), collie eye anomaly (CEA, OMIA 000218-9615), a 4-bp deletion in the canine *MDR1*/*ABCB1* gene associated with ivermectin toxicosis (IT, OMIA 001402-9615), degenerative myelopathy (DM, OMIA 000263-9615), congenital eye disease and deafness associated with merle coat (MER, OMIA 000211-9615), and selective cobalamin malabsorption (SCM, OMIA 001786-9615). Each associated mutations are described in Table 1. Saliva samples have been periodically collected from 478 Border Collies in Japan aged 2 months to 14 years. These samples were collected between 2006 and 2013 by the Japan Border Collie Health Network (JBCHN), a volunteer breeders' association with the owners' informed consent. Genotyping was carried out using previously reported real-time PCR methods, i.e., SYBR Green-based real-time PCR method with a melting curve analysis for CEA (Chang et al., 2010b) and TaqMan minor groove binder (MGB) probe-based real-time PCR methods for NCL, TNS, IT, and DM (Mizukami et al., 2011, 2012b, 2013b; Chang et al., 2013). As for SCM, TaqMan MGB probe-based real-time PCR method was established using the same protocol as described methods with forward (5'-ACT CGG ACC ATT CTG TGC AAA AT-3') and reverse (5'-CAC ATC AAT TTA AAA AGC GCA GAG AAT G-3') primers and allele specific probes (wild-type: 5'-CAT GGA ACA CAC AGA CTT-3', mutant: 5'-CAT GGA ACA CAA GAC TT-3'). The genotypes of MER were determined using length polymorphism (LP) analysis reported previously (Clark et al., 2006). However, because the LP analysis had the potential to

miss heterozygous carrier due to the difference in PCR amplification efficiency between wild-type and mutant allele, the mutant allele specific PCR amplification (307-base pair) using a new forward primer (5'-AGA CAC AGG CAG AGG GAG AA-3') and a reverse primer common with the LP analysis was complementally used for the purpose of more correct detection of heterozygous carrier. A simulation analysis was performed based on the allele frequencies in the genetic disorders examined in this study.

5.4. RESULTS

The results of each examination are described in Table 1. Data of NCL, TNS, CEA, and IT were the same as previous chapters. The frequencies of heterozygous carriers and mutant homozygotes of CEA were 25.3% and 1.9%, respectively. The frequencies of heterozygous carriers of NCL, TNS, IT, DM, MER, and SCM were 6.9%, 11.3%, 0.4%, 1.5%, 2.9% and 2.9%, respectively. There were no mutant homozygotes in these 6 genetic disorders. The ratio of dogs having 1 mutant allele was 35.2% and the ratio of dogs simultaneously having 2 and 3 different mutant alleles were 8.4% and 0.4%, respectively. 80% of dogs having 2 different mutant alleles were dogs having CEA. The combination of the 3 different mutant alleles was CEA, TNS, and MER. There were no dogs that simultaneously had more than 4 different mutant alleles. The resulting ratio of dogs without all 7 mutant alleles was 56.1%.

5.5. DISCUSSION

Generally, it is said that avoiding popular sire effects is the best way of preventing the dissemination of canine genetic disorders and trying to completely eliminate recessive disorders should be avoided because it may contribute to future loss of genetic diversity and the dissemination of new recessive disorders (Leroy and Baumung, 2013; Wade, 2011). Meanwhile, from the point of view of animal welfare, it is ideal that the population composed by dogs free from as many known genetic disorders as possible is retained because no affected dogs is born in the population. This study demonstrated that the size of the population equivalent to it was relatively large in the case of Border Collies (56.1%). This figure indicates that inbreeding rate does not increase so much in a sufficient effective population size even if most of heterozygous carriers and mutant homozygotes are excluded from breeding: increase of inbreeding rate is less than 2% when effective population size before selection is larger than approximately 45 (Fecht and Distl, 2008). To eliminate all genetic disorders at once should be impractical because effective population size is different from region to region and there are commercial issues of breeders. Therefore, we recommend that the prevention of multiple genetic disorders should be performed in turn or with different effort levels according to priority decided based on clinical significance including penetrance, prevalence, and clinical sign. For example, NCL and TNS have high priority due to high penetrance, relatively high prevalence, and lethal clinical course (Mizukami et al., 2012b; Shearman and Wilton, 2011). Meanwhile, IT has low priority because the prevalence is extremely low and mutant homozygotes of IT do not present with symptoms unless P-glycoprotein-mediated drugs was administered (Mizukami et al., 2012b). There is other

prevention measure that the mating between dogs having the identical mutant allele is banned and subsequently only puppies which are clear of all mutant alleles are selected for breeding. All breeding dogs have to take genetic tests in both measures and all puppies also have to take genetic tests in latter one. These prevention measures make it possible to mildly reduce both affected dogs and deleterious alleles with a minimum of loss of genetic diversity.

Fundamental data about each genetic disorder is needed to establish the prevention scheme. This study first clarified the accurate mutant allele frequency of DM and MER, which are relatively low. The mutant allele frequency of SCM in this report was half as high as that in the other report (Owczarek et al., 2013). This difference should result from population stratification effects due to geographical origin (Quignon et al., 2007). CEA was the most frequent genetic disorder in Border Collies. The reason should be that the lesion is confined to oculus and the phenotype has neutral or only weak effect on subsistence and selection. The carrier frequency of NCL in this study (6.9%) was decreased compared to that in the previous study (8.1%) (Mizukami et al., 2011). The major factor of this trend should be the prevention activity aggressively performed in tandem with JBCHN (Mizukami et al., 2012c). The similar prevention has to be applied to TNS as soon as possible because, to the best of our knowledge, the carrier frequency is the highest among canine fatal genetic disorders. It should take a considerable amount of time to complete the prevention of multiple genetic disorders. In addition, new genetic disorders will be found in the future. Therefore, continuous and patient prevention activity is needed to establish Border Collies' population with 'breed health'.

Table 9

Frequencies of mutant alleles associated with genetic disorders in Border Collies among 478 examined dogs.

Disorder	Mutation	Number of genotypes (%)		Frequency (%) of mutant allele
		Heterozygous carrier	Mutant homozygote	
NCL	c.619C>T in *CLN5* gene (Melville et al., 2005)	33 (6.9)	0 (0)	3.5
TNS	g.4411950_4411953delGTTT in *VPS13B* gene (Shearman and Wilton, 2011)	54 (11.3)	0 (0)	5.7
CEA	Intronic deletion of 7.8 k-bp in *NHEJ1* gene (Parker et al., 2007)	121 (25.3)	9 (1.9)	14.5
IT	c.227_230delAGAT in *MDR1*/*ABCB1* gene (Mealey et al., 2011)	2 (0.4)	0 (0)	0.2*
DM	c.118G>A in *SOD1* gene (Awano et al., 2009)	7 (1.5)	0 (0)	0.7
MER	SINE insertion in *SILV* gene (Clark et al., 2006)	14 (2.9)	0 (0)	1.5
SCM	c.8392delC in *CUBN* gene (Owczarek et al., 2013)	14 (2.9)	0 (0)	1.5

NCL, neuronal ceroid lipofuscinosis; TNS, trapped neutrophil syndrome; CEA, collie eye anomaly; IT, a 4-bp deletion in the canine *MDR1*/*ABCB1* gene associated with ivermectin toxicosis; DM, degenerative myelopathy; MER, congenital eye disease and deafness associated with merle coat; SCM, selective cobalamin malabsorption; SINE, short interspersed element.

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