NOTE *Internal Medicine*

Real-time PCR genotyping assay for canine progressive rod-cone degeneration and mutant allele frequency in Toy Poodles, Chihuahuas and Miniature Dachshunds in Japan

Moeko KOHYAMA[1)], Naomi TADA[2)], Hiroko MITSUI[2)], Hitomi TOMIOKA[2)], Toshihiko TSUTSUI[2)], Akira YABUKI[1)], Mohammad Mahbubur RAHMAN[1)], Kazuya KUSHIDA[1)], Keijiro MIZUKAMI[1)] and Osamu YAMATO[1)]*

[1)]*Laboratory of Clinical Pathology, Joint Faculty of Veterinary Medicine, Kagoshima University, 1–21–24 Kohrimoto, Kagoshima 890–0065, Japan*
[2)]*International Institute of Small Animal Medicine (Bio Plus), AHB Inc., 3–7–11 Kiba, Koutou-ku, Tokyo 135–0042, Japan*

(Received 11 May 2015/Accepted 22 October 2015/Published online in J-STAGE 6 November 2015)

ABSTRACT. Canine progressive rod-cone degeneration (PRCD) is a middle- to late-onset, autosomal recessive, inherited retinal disorder caused by a substitution (c.5G>A) in the canine *PRCD* gene that has been identified in 29 or more purebred dogs. In the present study, a TaqMan probe-based real-time PCR assay was developed and evaluated for rapid genotyping and large-scale screening of the mutation. Furthermore, a genotyping survey was carried out in a population of the three most popular breeds in Japan (Toy Poodles, Chihuahuas and Miniature Dachshunds) to determine the current mutant allele frequency. The assay separated all the genotypes of canine PRCD rapidly, indicating its suitability for large-scale surveys. The results of the survey showed that the mutant allele frequency in Toy Poodles was high enough (approximately 0.09) to allow the establishment of measures for the prevention and control of this disorder in breeding kennels. The mutant allele was detected in Chihuahuas for the first time, but the frequency was lower (approximately 0.02) than that in Toy Poodles. The mutant allele was not detected in Miniature Dachshunds. This assay will allow the selective breeding of dogs from the two most popular breeds (Toy Poodle and Chihuahua) in Japan and effective prevention or control of the disorder.

KEY WORDS: canine *PRCD* gene, mutant allele frequency, progressive rod-cone degeneration, real-time PCR genotyping, retinal disorder

doi: 10.1292/jvms.15-0279; *J. Vet. Med. Sci.* 78(3): 481–484, 2016

Canine progressive retinal atrophy (PRA) is a hereditary retinal disorder that includes a group of conditions with similar clinical presentation, although the age of onset and rate of progression vary considerably by breed [10]. PRA has been divided into various forms based on the age of onset, pathologic features and genetic mutations in certain genes [5, 10].

Progressive rod-cone degeneration (PRCD) is one of forms in canine PRA, which is a middle- to late-onset, autosomal recessive retinal disorder causing photoreceptor degeneration [6, 10]. It has been reported that a point mutation c.5G>A (p.Cys2Tyr) in the canine *PRCD* gene is associated with PRCD in 29 or more breeds: American Cocker Spaniel, American Eskimo Dog, Australian Cattle Dog, Australian Shepherd, Australian Shepherd (Miniature), Australian Stumpy Tail Cattle Dog, Chesapeake Bay Retriever, Chinese Crested, Cockapoos, Dwarf Poodle, English Cocker Spaniel, Entelbucher Mountain Dog, Finish Lapphund, Golden Retriever, Golden Doodle, Karelian Bear Dog, Kuvasz, Labradoodle, Labrador Retriever, Lapponian Herder, Markiesje, Moyen Poodle, Norwegian Elkhound, Nova Scotia Duck Tolling Retriever, Poodle (Miniature and Toy), Portuguese Water Dog, Spanish Water Dog, Swedish Lapphund, Yorkshire Terrier [2, 3, 11] and other breeds (Optigen, http://www.optigen.com/). However, to our knowledge, the carrier rate and/or mutant allele frequency in each breed have not been clarified in Japan.

According to the Japan Kennel Club (http://www.jkc.or.jp/), a certified club by the Federation Cynologique Internationale (http://www.fci.be/), Poodles, Chihuahuas and Dachshunds have been the three most popular breeds in Japan. From 2003 to 2014, the annual number of these three breeds has accounted for 48 to 57% of the annual total number of all the registered purebred dogs. Therefore, it is very important to know the current carrier rate and/or mutant allele frequency of canine PRCD in these three popular breeds, in order to apply preventive measures for this ocular disorder. In addition, an easy and reliable genotyping method is required for screening the disorder.

In the present study, a TaqMan probe-based real-time PCR genotyping assay was developed and evaluated for rapid genotyping and large-scale screening for the mutation of canine PRCD. Furthermore, a genotyping survey was carried out in a randomly selected population of Toy Poodles, Chihuahuas and Miniature Dachshunds.

All experimental procedures using animal samples were performed in accordance with the guidelines regulating the animal use at Kagoshima University. Whole blood samples of 200 Toy Poodles, 57 Chihuahuas and 100 Miniature

*CORRESPONDENCE TO: YAMATO, O., Laboratory of Clinical Pathology, Joint Faculty of Veterinary Medicine, Kagoshima University, 1–21–24 Kohrimoto, Kagoshima 890–0065, Japan.
e-mail: osam@vet.kagoshima-u.ac.jp

Table 1. Primers and probes used in the real-time PCR assay for rapid genotyping and large-scale screening for the mutation of canine progressive rod-cone degeneration

Primer/probe	Sequence 5′→3′ (mer)	Reporter (5′)	Quencher (3′)	Tm (°C)	Concentration (nM)
Forward primer	CCTTTCTCCTGCAGACTCTGT (21)	NA	NA	56.5	450
Reverse primer	CCAAGGTGCTGAGTAGGAAGAG (22)	NA	NA	57.0	450
Probe for wild-type allele	TGAGCCATGTGCACCAC (17)	VIC	NFQ	55.8	100
Probe for mutant allele	TGAGCCATGTACACCAC (17)	FAM	NFQ	51.8	100

Tm: melting temperature calculated by OligoAnalizer 3.1 (https://sg.idtdna.com/calc/analyzer). NA: not applicable. VIC: 6-carboxyrhodamine. FAM: 6-carboxyfluorescein. NAQ: nonfluorescent quencher. Underlined letter in the sequence of the probe indicates the corresponding guanine to a substitution mutation (c.5G>A) in the canine *PRCD* gene.

Dachshunds, without any known ophthalmic diseases, were randomly collected from several animal hospitals in Japan with the cooperation of their owners and veterinarians. The whole blood samples were spotted onto Flinders Technology Associates filter papers (FTA card; Whatman International Ltd., Piscataway, NJ, U.S.A.) and stored until use. Control DNA samples from dogs (three wild-type, three carrier and one mutant homozygous) were used after the confirmation of each genotype by direct DNA sequencing.

For the real-time PCR assay, primers and TaqMan minor groove binder (MGB) probes were designed based on the sequences of the canine *PRCD* gene in a wild-type dog (GenBank accession numbers NC_006591.3 and NM_001097560.1) and a dog possessing the PRCD-associated mutation (c.5G>A). These primers and probes (Table 1) were synthesized by a commercial company (Applied Biosystems, Foster City, CA, U.S.A.) and bound to a fluorescent reporter dye (6-carboxyrhodamine or 6-carboxyfluorescein) at the 5′-end and a nonfluorescent quencher dye at the 3′-end. DNA extraction was performed as previously described using a disc that punched out of each FTA card [9]. The amplifications were carried out using the StepOne real-time PCR system (Applied Biosystems) in a final volume of 10 μl consisting of 2 × PCR master mix (TaqMan GTXpress Master Mix, Applied Biosystems), 80 × genotyping assay mix (TaqMan SNP Genotyping Assays, Applied Biosystems) containing the specific primes and TaqMan MGB probes and template DNA. Prior to PCR, the prepared mixtures were held at 25°C for 30 sec. The cycling conditions consisted of 20 sec at 95°C, followed by 60 cycles of 3 sec at 95°C and 20 sec at 63°C. The holding stage after PCR was carried out at 25°C for 30 sec. In the process of seeking the optimal annealing temperature (63°C), several annealing temperatures (60–64°C) were evaluated to differentiate the amplifications of wild-type alleles from those of mutant alleles. The obtained data were analyzed with StepOne version 2.3 (Applied Biosystems), and the calculations were based on the results obtained from DNA samples of control dogs.

The real-time PCR assay clearly separated all three genotypes at the annealing temperature of 63°C (Fig. 1). The total required time for the 60-cycle amplification was less than 1 hr. At the annealing temperature of 60–62°C, nonspecific allelic amplification was observed in the wild-type allele, because false mutant-allelic amplification occurred together with the true wild-type-allelic amplification (Fig. 2). The false mutant-allelic amplification was inhibited moderately

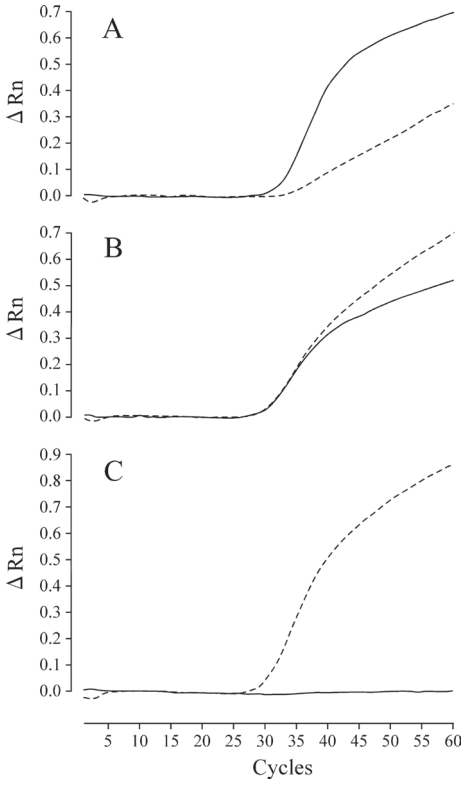

Fig. 1. Real-time PCR amplification plots of wild-type and mutant alleles in canine progressive rod-cone degeneration. Amplification was plotted as fluorescence intensity (ΔRn value) against cycle number. The ΔRn value is the reporter dye signal normalized to the internal reference dye and corrected for the baseline signal established in the first few cycles of PCR. Each of the three amplification plots shows the homozygous wild-type (A), heterozygous carrier (B) and homozygous mutant genotypes (C) at the annealing temperature of 63°C. Solid and dotted lines indicate amplification of wild-type and mutant alleles, respectively.

by an increase in the annealing temperature at 63°C and 64°C, but fluorescence intensity decreased markedly at 64°C compared to that at 60–63°C (Figs. 1 and 2). The quantification cycles were delayed as the annealing temperature was raised (Fig. 2). Consequently, an annealing temperature of 63°C was considered optimal for this method at which it was possible to clearly distinguish the allelic types during

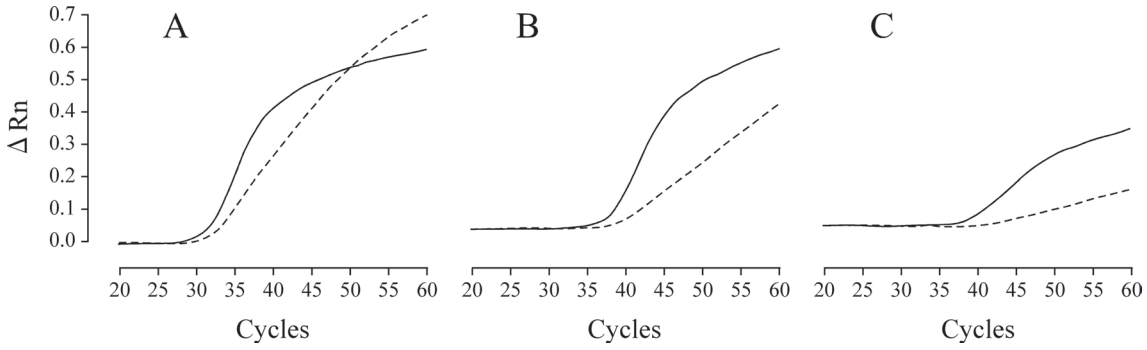

Fig. 2. Real-time PCR amplification plots of homozygous wild-type genotype in canine progressive rod-cone degeneration. Amplification plots were obtained at three different annealing temperatures of 60°C (A), 62°C (B) and 64°C (C). Solid and dotted lines indicate amplification of true wild-type and false mutant alleles, respectively.

Table 2. The rate of carriers and dogs with risk for progressive rod-cone degeneration and the allele frequency for canine PRCD-associated mutation in the three most popular breeds (Toy Poodle, Chihuahua and Miniature Dachshund) in Japan

Dog breed	Number of examined dogs	Number of carriers (rate,%)	Number of risk* (rate,%)	Mutant allele frequency	95% confidence interval of mutant allele frequency
Toy Poodle	200	33 (16.5)	1 (0.5)	0.088	0.062–0.120
Chihuahua	54	2 (3.7)	0 (0)	0.019	0.002–0.065
Miniature Dachshund	100	0 (0)	0 (0)	0	ND

* Risk means the possibility of progressive rod-cone degeneration in a homozygous mutant genotype; however, the dog that had the risk could not be followed up clinically. ND: not determined.

the 60-cycle amplification, although a low nonspecific allelic amplification was also observed.

The results of the survey among the three most popular purebred dogs in Japan are shown in Table 2. The survey demonstrated that the carrier rate in Toy Poodles was 16.5% and the mutant allele frequency was 0.088. We identified a dog that was homozygous for the mutation and had a risk for canine PRCD, but the dog could not be followed up clinically. The survey also demonstrated that the carrier rate in Chihuahuas was 3.7% and the mutant allele frequency was 0.019. The mutant allele was not detected in Miniature Dachshunds.

In the present study, a real-time PCR assay using TaqMan MGB probes was developed to provide clear-cut genotyping results for the wild-type and mutant alleles associated with canine PRCD (Fig. 1). The use of FTA cards for sampling that eliminated the need for traditional multi-step DNA extraction and purification procedures [9], combined with the relatively short amplification time (less than one hour), allowed rapid genotyping and screening of this mutation in less than 2 hr. However, there was a slight nonspecific false allelic amplification in this assay. The difference in the amplification plots of the wild-type and mutant alleles was probably caused by the difference in the melting temperature (Tm) of each probe (Table 1) [4]. Although the Tm values of both probes seem to increase by the addition of an MGB moiety, false mutant-allelic amplification occurred at the standard annealing temperature of 60°C. In the present study, increasing the annealing temperature to 63°C improved the reliability of the assay, although it decreased the fluorescent intensity and delayed the quantification cycles to a small extent. Overall, the data showed that the proposed assay is a simple, rapid and reliable tool for small- and large-scale surveys and will help prevention and control of canine PRCD.

Regarding the genotyping assays for canine PRCD, conventional PCR assays coupled with digestion, using two kinds of restriction endonucleases, were developed previously in which the PRCD-associated mutation was identified [11]. Subsequently, a real-time PCR using locked nucleic acid (LNA) TaqMan probes was also developed as a more rapid method to detect the mutation [3]. Similar to these previous assays, the real-time PCR assay with TaqMan MGB probes developed in the present study also provides distinct genotyping results. There seems to be no major differences in performance between LNA and MGB probes, except for the duration of the run [1]. However, the LNA probes-based assay seems to require a longer run duration than the MGB probes-based assay as shown in the protocols. In addition, the use of FTA cards is also one of the advantages in our method to further reduce the total time required (less than two hours).

The survey demonstrated that the mutant allele frequency in Toy Poodles, calculated from 33 carriers and one homozygous mutant genotype in a population of 200 randomly sampled dogs, was approximately 0.09 (Table 2). This frequency is considered high enough to allow the establishment of measures for the control of the disorder in Toy Poodles in Japan. In addition, the mutant allele was detected for the first

time in Chihuahuas, and the frequency was approximately 0.02. Although the mutant allele frequency was lower than that in Toy Poodles, it is still high enough to allow the establishment of measures for the control of the disorder in Chihuahuas in Japan. The disease control based on clinical signs is difficult as an electroretinogram measurement and a fundoscopic examination are required to identify the early stages of PRA, before clinical blindness is evident. Furthermore, a carrier status cannot be determined using existing clinical techniques. The mutant allele frequency in Toy Poodles and Chihuahuas could be decreased and/or controlled, if the genotyping test was used at breeding kennels. Genotyping and selection could easily prevent the breeding of dogs that have a risk of PRCD or the breeding between carriers and thereby the production of dogs with risk for PRCD in the future, while preserving the gene pool in each breed. Genotyping should be used for breeding and diagnostic purposes, but not casually for the healthy client-owned dogs, which are not intended for breeding especially considering the financial burden on the owner without offering any preventive benefits.

In the present study, the mutant allele was not detected in any of the 100 randomly collected Miniature Dachshunds (Table 2), suggesting that the mutant allele of canine PRCD is unlikely to be present in Miniature Dachshunds in Japan. Therefore, based on the present sample population, PRCD genotyping in this breed is considered less critical. However, canine cone-rod dystrophy is known to be prevalent in Miniature Dachshunds as another form of canine inherited retinal degeneration, which is mainly associated with a certain mutation in the canine *RPGRIP1* gene [7, 8]. The prevention and control of inherited canine retinal degeneration in Miniature Dachshunds should be considered based on this mutation. The total control of canine retinal disorders should be planned based on specific data for each breed in each country.

In conclusion, the present study clarified the prevalence of canine PRCD in the three most popular purebred dogs in Japan and demonstrated that the mutant allele frequency was high enough to allow the establishment of measures for the prevention and control of the disorder in Toy Poodles and Chihuahuas. Selective breeding of dogs from these two breeds can be achieved using a reliable genotyping test, such as the TaqMan probe-based real-time PCR genotyping assay developed in this study.

ACKNOWLEDGMENTS. The authors are grateful to Mr. Yutaka Okada of AHB Inc. for his advice. This study was supported by the Ministry of Education, Culture, Sports, Science and Technology of Japan (MEXT grants nos. 25292181 and 26660242 to OY).

REFERENCES

1. Buh Gašparič, M., Tengs, T., La Paz, J. L., Holst-Jensen, A., Pla, M., Esteve, T., Žel, J. and Gruden, K. 2010. Comparison of nine different real-time PCR chemistries for qualitative and quantitative applications in GMO detection. *Anal. Bioanal. Chem.* **396**: 2023–2029. [Medline] [CrossRef]
2. Downs, L. M., Hitti, R., Pregnolato, S. and Mellersh, C. S. 2014. Genetic screening for PRA-associated mutations in multiple dog breeds shows that PRA is heterogeneous within and between breeds. *Vet. Ophthalmol.* **17**: 126–130. [Medline] [CrossRef]
3. Gentilini, F., Rovesti, G. L. and Turba, M. E. 2009. Real-time detection of the mutation responsible for progressive rod-cone degeneration in Labrador Retriever dogs using locked nucleic acid TaqMan probes. *J. Vet. Diagn. Invest.* **21**: 689–692. [Medline] [CrossRef]
4. Kutyavin, I. V., Afonina, I. A., Mills, A., Gorn, V. V., Lukhtanov, E. A., Belousov, E. S., Singer, M. J., Walburger, D. K., Lokhov, S. G., Gall, A. A., Dempcy, R., Reed, M. W., Meyer, R. B. and Hedgpeth, J. 2000. 3′-Minor groove binder-DNA probes increase sequence specificity at PCR extension temperatures. *Nucleic Acids Res.* **28**: 655–661. [Medline] [CrossRef]
5. Mellersh, C. S. 2014. The genetics of eye disorders in the dog. *Canine Genet. Epidemiol.* **1**: 3. [Medline] [CrossRef]
6. Miyadera, K., Acland, G. M. and Aguirre, G. D. 2012. Genetic and phenotypic variations of inherited retinal diseases in dogs: the power of within- and across-breed studies. *Mamm. Genome* **23**: 40–61. [Medline] [CrossRef]
7. Miyadera, K., Kato, K., Boursnell, M., Mellersh, C. S. and Sargan, D. R. 2012. Genome-wide association study in *RPGRIP1*$^{-/-}$ dogs identifies a modifier locus that determines the onset of retinal degeneration. *Mamm. Genome* **23**: 212–223. [Medline] [CrossRef]
8. Miyadera, K., Kato, K., Aguirre-Hernandez, J., Tokuriki, T., Morimoto, K., Busse, C., Barnett, K., Holmes, N., Ogawa, H., Sasaki, N., Mellersh, C. S. and Sargan, D. R. 2009. Phenotypic variation and genotype-phenotype discordance in canine cone-rod dystrophy with an *RPGRIP1* mutation. *Mol. Vis.* **15**: 2287–2305. [Medline]
9. Mizukami, K., Chang, H.S., Yabuki, A., Kawamichi, T., Kawahara, N., Hayashi, D., Hossain, M. A., Rahman, M. M., Uddin, M. M. and Yamato, O. 2011. Novel rapid genotyping assays for neuronal ceroid lipofuscinosis in Border Collie dogs and high frequency of the mutant allele in Japan. *J. Vet. Diagn. Invest.* **23**: 1131–1139. [Medline] [CrossRef]
10. Petersen-Jones, S. M. 2014. Canine Retinopathies. pp. 1188–1192. *In*: Kirk's Current Veterinary Therapy, 15th ed. (Bonagura, J. D. and Twedt, D. C. eds.), Elsevier, St. Louis.
11. Zangerl, B., Goldstein, O., Philp, A. R., Lindauer, S. J. P., Pearce-Kelling, S. E., Mullins, R. F., Graphodatsky, A. S., Ripoll, D., Felix, J. S., Stone, E. M., Acland, G. M. and Aguirre, G. D. 2006. Identical mutation in a novel retinal gene causes progressive rod-cone degeneration (*prcd*) in dogs and retinitis pigmentosa in man. *Genomics* **88**: 551–563. [Medline] [CrossRef]