

Efficient screening of the cystinuria-related C663T *Slc3a1* nonsense mutation in Newfoundland dogs by denaturing high-performance liquid chromatography

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Abstract. Cystinuria in Newfoundland dogs is a metabolic disease associated with a nonsense mutation in the exon 2 of the *Slc3a1* gene. Similar to type I human cystinuria, heterozygote carriers are not affected by the disease and do not reveal differences in urinary concentration of dibasic amino acids when compared with normal dogs. However, through a recessive mode of inheritance, these dogs are able to transmit the disease to their offspring. Early detection of mutation carriers through cost-effective reliable methods is therefore essential for the implementation of breeding methods aimed at the eradication of the disease. Denaturing high-performance liquid chromatography (DHPLC) is a recently developed technique for rapid and efficient screening of nucleotide polymorphisms in polymerase chain reaction–amplified products. This technique was used for the identification of the C663T *Slc3a1* mutation in Portuguese Newfoundland dogs. Polymerase chain reaction products amplified from a region containing the C663T locus were subjected to DHPLC analysis, and results were double checked by DNA sequencing. Results showed the presence of the mutation in 6 of the 22 dogs tested. Urine biochemical parameters correlated well with the number of mutated *Slc3a1* copies, and homozygotes for the C663T mutation were the only dogs diagnosed with cystinuria. Sequence analysis confirmed the DHPLC results, demonstrating that the technique could be a reliable alternative to sequencing for the rapid and cost-effective identification of mutations in canine breeds.

Key words: Cystinuria; denaturing high-performance liquid chromatography; mutation screening; Newfoundland dogs.

Cystinuria is a metabolic disease caused by an inherited defect of renal reabsorption of dibasic amino acids (lysine, arginine, ornithine, and cystine).¹⁰ It is one of the most common human congenital defects and causes a malfunction of renal tubular transport proteins.⁷ It has been described in more than 60 dog breeds; however, as in humans, it appears to be a heterogeneous disease.⁹ In affected human patients, 3 forms of the disease have been described: type I, caused by mutations in the *SLC3A1* gene, and types II and III, related to mutations in the *SLC7A9* gene.^{2,4} A severe form of canine cystinuria, resembling type I human form, has been recognized in Newfoundland dogs. In 1995, Casal et al.³ studied 2 families of Newfoundland dogs where the disease was prevalent and concluded that the disease had an autosomal recessive mode of inheritance and that obligate heterozygotes did not have clinical signs or abnormal amino acid urinary profiles. More recently, Henthorn et al.⁵ identified a nonsense mutation at nucleotide 111 in exon 2 of the *Slc3a1* gene in cystinuric Newfoundland dogs, a defect that was not found in dogs of other breeds

affected by the disease. The mutation causes the replacement of an arginine residue with a stop codon, resulting in the translation of a severely truncated polypeptide. This recognition allowed the development of nucleic acid–based diagnostic tests for early detection of Newfoundland mutation carriers.

Denaturing high-performance liquid chromatography (DHPLC) has been established as an alternative to DNA sequencing for detection of DNA sequence variation.^{7,13} In comparison with other methods for DNA mutation screening, the WAVE DHPLC has been consistently reported as being more sensitive, faster, and more cost effective,^{1,11,12} thus representing the ideal technology for screening genomic samples for sequence variation.

This study describes the development of a DHPLC technique for detection of a nonsense mutation in the exon 2 of the *Slc3a1* gene in Newfoundland dogs and the application of this technique to the screen for the mutation in a population of Portuguese Newfoundland dogs.

Twenty-two Newfoundland dogs (6 males, 16 females; age 2 months to 8.8 years, mean age 3.1 years) were enrolled in the study by voluntary decision of the owners or breeders. Only 1 male dog had a history of cystine urolithiasis, whereas the other 21 dogs were considered healthy animals. Genomic DNA was extracted from a 5-ml whole blood sample collected through jugular venipuncture. Blood was collected in EDTA-coated containers and frozen at -20°C until

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processing. Genomic DNA was extracted from blood by the “salting out” method.⁸ Briefly, 1.5 ml of blood was mixed with 1.5 ml of a 0.9% NaCl solution in polypropylene centrifuge tubes and centrifuged at $16,000 \times g$ for 20 minutes. After the supernatant was discarded, 1.4 ml of ACE buffer (0.3M NH_4Cl , 3.4 mM EDTA, 1.6 mM KH_2PO_4) was added to the pellet and shaken vigorously for 15 seconds, followed by agitation in an orbital shaker for 30 minutes (at 4°C) and centrifugation at $16,000 \times g$ for 20 minutes. After this step was performed twice, cells were digested overnight at 56°C with 600 μl of Nucleic Lysis Buffer^a (40 μl of 10% SDS and 12 μl of 20 mg/ml proteinase K). Ammonium acetate (0.2 ml; 11.6 M) was added to each tube and shaken vigorously for 15 seconds, followed by centrifugation at $16,000 \times g$ for 20 minutes at 4°C . Supernatant was transferred to a fresh tube, and DNA was precipitated with ice-cold 100% ethanol, followed by centrifugation at $16,000 \times g$ for 10 minutes. The DNA was washed with 100 μl 70% ethanol for 10 minutes and air dried at room temperature for 10 minutes, and the pellet was removed with a pipette and transferred to a sterile 1.5-ml microcentrifuge tube containing 0.1 ml H_2O . The DNA was allowed to dissolve overnight at 4°C before use.

Five microliters of DNA was used for PCR amplification of a 171-bp *Slc3a1* amplicon containing the C663T locus by using the SLC3A1Ex2F (5'-CAA-GAGAACTGGACTACATC-ACA-3') and SLC3A1Ex2R (5'-ATCGTGTATGGCTGCAAGCAGA-3') oligonucleotides as primers and the Expand High Fidelity PCR System.^b Amplification was performed in a GeneAmp PCR System 9700^c with an initial denaturation step of 3 minutes at 95°C , followed by 30 cycles at 94°C (1 minute), 60°C (45 seconds), and 72°C (45 seconds) and a final extension step of 7 minutes at 72°C .

For DHPLC analysis, either 10 μl of each sample alone or 5 μl of each sample mixed (1:1) with a wild-type (WT) control were heat denatured at 95°C for 5 minutes and slowly cooled to 25°C for 45 minutes in a TGradient thermocycler^d to allow for the formation of heteroduplexes. Analysis was carried out by the 3500A WAVE DNA Fragment Analysis System.^e Seven microliters of each PCR product was loaded onto a reversed-phase DNASep column^e preheated at 55.3°C . Hetero- and homoduplexes were loaded with 56% Buffer A^e (0.1 mol/liter triethylamine acetate, pH 7.0) and 44% Buffer B^e (0.1 mol/liter triethylamine acetate, pH 7.0, containing 25% [v/v] acetonitrile) and eluted with a linear acetonitrile gradient of Buffer A (Start 51%; Stop 42%) and Buffer B (Start 49%; Stop 58%) for 4.5 minutes at a constant flow rate of 0.9 ml/minute. Before each set of injections, the column was equilibrated at starting conditions for 3 minutes and 2

Table 1. Comparison of costs (€) between mutation detection by denaturing high-performance liquid chromatography (DHPLC) and DNA sequencing analysis (VAT included).

Detection method	Equipment cost	Thermocycler cost	Polymerase chain reaction cost	Sample preparation and analysis costs
DNA sequencing*	180,000	5,000	2.8	17.2
DHPLC†	83,000	5,000	2.8	1.8

* ABI Prism 3100 Avant (16 capillaries).

† Transgenomic 3500A.

blank (no DNA) injections were performed to guarantee for the maximum resolution in mutation detection.

Urine samples were also analyzed for presence of amino acids. Briefly, samples were individually diluted to a 1 mmol/liter creatinine concentration. Filter paper^f Grade 903 was soaked with the urine sample. A single 3.2-mm dried urine spot was placed in a polypropylene 96-well plate, and 100 μl of methanolic internal standard solution was added. The deuterium-labeled internal standards used were [$^2\text{H}_2$]-ornithine ([$^2\text{H}_2$]-Orn) and [$^2\text{H}_4$]-[$^{13}\text{C}_1$]-arginine ([$^2\text{H}_4$]-[$^{13}\text{C}_1$]-L-Arg). The plate was gently shaken during the 20-minute extraction of the amino acid markers. The methanol extract was then manually transferred to a second polypropylene 96-well plate and dried with a hot-air blower. Butanol-HCl (60 ml) was placed in each sample well, and the plate was incubated in a 65°C forced-air oven for 15 minutes. Samples were then reconstituted with 100 μl of acetonitrile and water (50:50 v:v plus 0.025% formic acid), and each plate was covered with aluminum foil. The samples were then analyzed by tandem mass spectrometry. The amino acids arginine and ornithine were analyzed with multiple-reaction monitoring.

Of the 22 Newfoundland specimens analyzed by DHPLC for the presence of the C663T *Slc3a1* mutation, 16 WTs, 5 heterozygotes, and 1 homozygote were found. These results were double checked by sequencing of the PCR-derived amplicons of the homozygote, all heterozygotes, and 5 randomly selected WT individuals. The sequencing results matched the DHPLC results (Fig. 1). The results obtained by the 2 techniques were in full agreement with the genealogic tree, thus establishing the DHPLC technique as a valid technique for the routine screening of the C663T *Slc3a1* mutation. The DHPLC technique proved to be accurate (full agreement with results from DNA sequencing), fast (results were obtained 6 minutes after loading), and inexpensive (€4.6 per sample, including PCR amplification), comparing favorably in terms of cost per

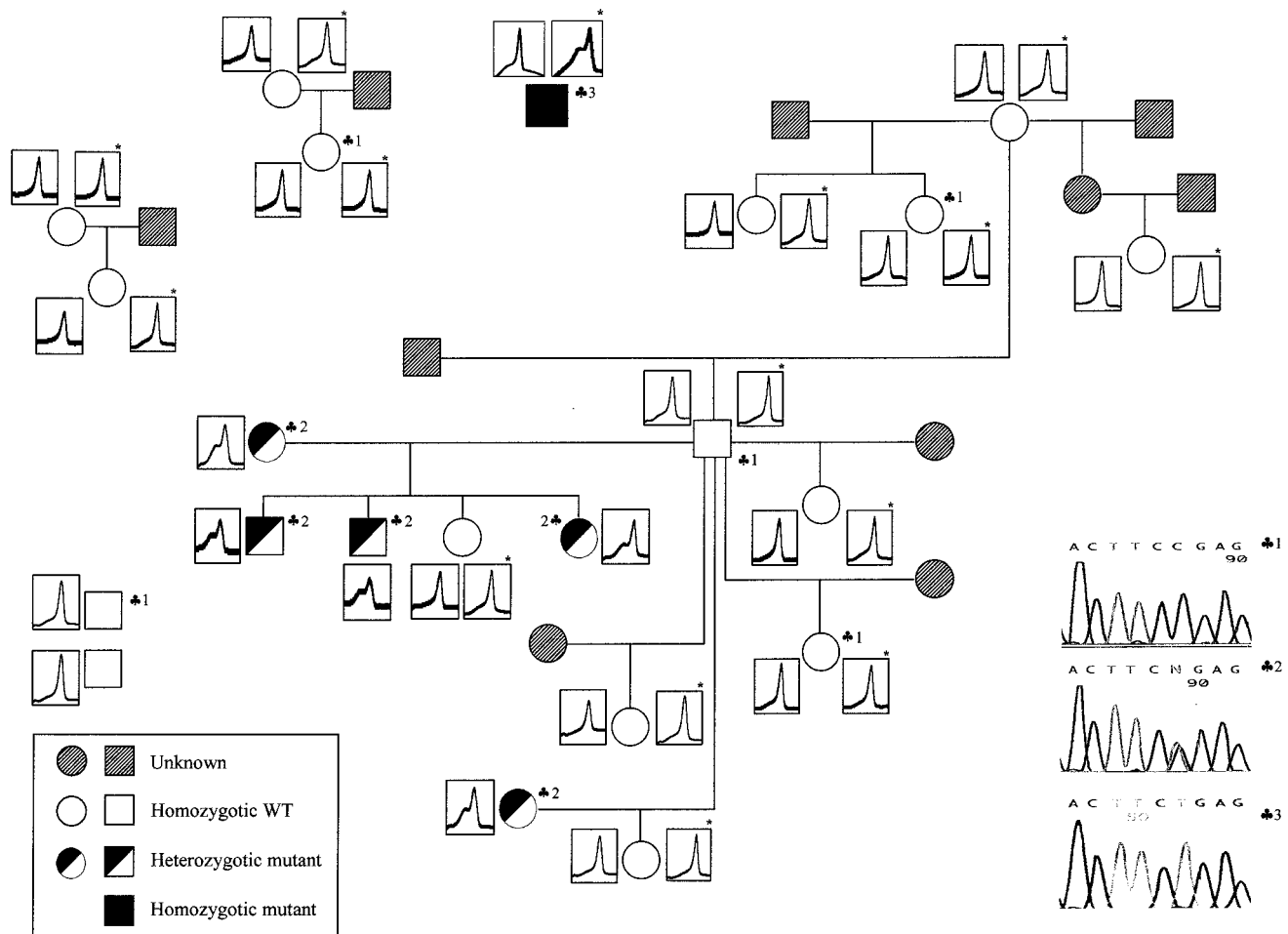


Figure 1. Genealogic trees of the Newfoundland dogs tested by denaturing high-performance liquid chromatography (DHPLC) for the presence of the *Slc3a1* C663T mutation. Males and females are represented by squares and circles, respectively. Homozygotic specimens are denoted by filled symbols, heterozygotic by half-filled symbols, wild type (WT) by empty symbols, and unknown specimens (not tested) by stripe-filled symbols. For each specimen studied, the DHPLC result is shown for the tests performed either with (indicated with an asterisk for WT or mutant homozygotes) or without (for heterozygotes) 1:1 mixing with the WT control. ♣1, 2, and 3 indicate the WT, mutant heterozygous, and mutant homozygous samples, respectively, analyzed by DNA sequencing. The DNA sequence spanning the mutation locus in each type of amplicon is shown.

sample with other genotyping technologies such as DNA sequencing (Table 1).

Detection of amino acids in urine samples collected from WT and heterozygote dogs revealed no traces of the dibasic amino acids arginine and ornithine in any of the urine samples, confirming the recessive mode of transmission of this disease. The homozygote result was obtained from the dog with a history of cystine urolithiasis. In addition, when the 663 locus of 4 non-Newfoundland dogs diagnosed with urinary cystine calculus was analyzed, no mutations were found. This is in agreement with the results of Henthorn et al.,⁵ who did not find the C663T *Slc3a1* mutation in dogs of other breeds affected by the disease.

In conclusion, the results of this study show that DHPLC can be used to efficiently detect the C663T

Slc3a1 mutation. Genotyping with this technology proved to be reliable, fast, and inexpensive, thus offering an attractive alternative to classic genotyping methods. However, it should be noted that the results herein are limited to the genetic screening of the only known cystinuria-related mutation in Newfoundland dogs. The limited number of animals investigated in this report and in a previous study⁵ is insufficient to rule out the possibility that cystinuria may be related to other mutations in a minority of Newfoundland dogs. It is also evident that other cystinuria-related mutations must be present in non-Newfoundland dog breeds. Further studies are needed to investigate the cystinuria-related mutation in more Newfoundland dogs and to expand the DHPLC technique to study cystinuria-related mutations in other canine breeds.

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Sources and manufacturers

- a. Sigma-Aldrich, St. Louis, MO.
- b. Roche Diagnostics GmbH, Penzberg, Germany.
- c. Applied Biosystems, Foster City, CA.
- d. Biometra GmbH i. L., Goettingen, Germany.
- e. Transgenomic, Inc., Omaha, NE.
- f. Schleicher and Schuell BioScience GmbH, Dassel/Relliehausen, Germany.

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