Evaluation of haplotypes associated with copper toxicosis in Bedlington Terriers in Australia

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Objective—To evaluate the haplotype distribution associated with the copper toxicosis gene and the segregation of the mutated allele in a Bedlington Terrier population in Australia.

Animals—131 Bedlington Terriers.

Procedure—Samples of DNA and RNA were obtained from each dog. Genetic status of each dog was evaluated by use of the DNA markers C04107; single nucleotide polymorphism (SNP), which was adjacent to exon 2 of *Murr1*; and a deletion marker for exon 2. A subgroup of the study population was evaluated by use of biochemical and histologic techniques to elucidate the correlation between genotype and phenotype.

Results—We identified a recombination between the C04107 marker and *Murr1* and a variation in a nucleotide in the splice site of exon 2 in our Bedlington Terrier cohort. Furthermore, we identified a novel haplotype associated with copper toxicosis in this cohort.

Conclusions and Clinical Relevance—Our findings indicate that the deletion of exon 2 was not the sole cause of copper toxicosis, although only exon 2 deletion of *Murr1* has been responsible for copper toxicosis in Bedlington Terriers. Although we failed to find a novel mutation in our cohort, we identified an affected dog family with an intact exon 2. Furthermore, we found that an SNP in the 5′ splicing site of exon 2 may or may not be associated with a novel mutation of the *Murr1* gene or other genes. Loss of linkage between the C04107 marker and the *Murr1* gene was also identified in a certain family of dogs. (*Am J Vet Res* 2004;65:1573–1579)

Inherited copper toxicosis in Bedlington Terriers is an autosomal recessive disease[1] that was first reported in 1975.[2] Copper toxicosis has also been reported in other dog breeds including Doberman Pinschers,[3,4] West Highland White Terriers,[5] Skye Terriers,[6] and Dalmatians,[7] as well as mixed-breed dogs.[8] Copper toxicosis in Bedlington Terriers has been proposed as a possible disease that could be useful for investigating copper storage diseases in humans, particularly Wilson disease.[9]

Copper toxicosis in Bedlington Terriers is characterized by increased hepatic concentrations of copper in association with reduced biliary excretion of stored copper.[10] Because the initial clinical signs in Bedlington Terriers with copper toxicosis are usually inconclusive, a definitive diagnosis has traditionally been made on the basis of hepatic copper concentrations and concurrent histopathologic changes. However, this is not always accurate for dogs < 1 year of age or dogs fed a diet low in copper. Also, the higher mean hepatic concentration of copper in Bedlington Terriers, compared with hepatic concentrations in other breeds of dogs, and the progressive nature of this disease can result in false-positive or false-negative diagnoses. Therefore, it may be difficult to establish a definitive diagnosis on the basis of traditional diagnostic criteria.

Prior to the identification of the *CT-BT* gene, a linkage-based DNA marker (ie, C04107) was used, with or without other diagnostic tests, to discriminate dogs affected with the *CT-BT* gene.[11-14] However, there may be loss of linkage between the C04107 marker and the *CT-BT* gene.[11,15,16] Therefore, the diagnostic value of the C04107 marker is limited when performed on unrelated individuals and without pedigree information.[17]

Until recently, it was believed that deletion of the entire exon 2 of the *Murr1* gene, as determined by use of linkage disequilibrium mapping, was solely responsible for copper toxicosis in the Bedlington Terrier cohort.[18] This provided a method of screening for affected Bedlington Terriers. However, not all Bedlington Terriers with copper toxicosis had the exon 2 deletion mutation. The genetic diagnosis for this breed was further complicated by the finding of a single nucleotide variation on the 5′ splice site of exon 2.[15]

In the study reported here, we investigated the haplotype distribution associated with the *CT-BT* gene and the segregation of the mutated allele in Bedlington Terriers in an Australian cohort. The results will enable us to develop a more reliable and accurate method of predicting copper toxicosis in dogs.

Materials and Methods

Animals—A population of 131 Bedlington Terriers from 8 pedigrees was included in the study. A subgroup of the study population was evaluated by use of biochemical and histologic techniques to elucidate the correlation between

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genotype and phenotype. Informed consent for the involvement of the dogs in this research project was obtained from the owners prior to participation of the dogs in the study.

Sample collection—A blood sample (11 mL) was collected from each dog and immediately placed into tubes that contained EDTA for hematologic evaluation (1 mL), lithium heparin for biochemical analysis (4 mL), and sodium citrate for coagulation testing (2 mL). The remaining 4 mL was placed in an EDTA tube for genetic analysis.

Conventional diagnostic tests—The methods used for hematologic examination, blood biochemical analysis, measurement of hepatic copper content, histologic examination, histochemical analysis, and electron microscopy have been described elsewhere.[19]

Extraction and amplification of DNA—Extraction of DNA was performed in accordance with the method of Lahiri and Nurnberger.[20] Following extraction, the quantity of DNA samples was determined by use of the absorption at a wavelength of 260 nm, and purity of DNA samples was determined by use of the ratio for the absorption at a wavelength of 260 nm to the absorption at a wavelength of 280 nm. The DNA samples were then diluted to a working concentration of 20 ng/mL. A **polymerase chain reaction (PCR)** assay was used to amplify the C04107 microsatellite marker, as described elsewhere.[14] Three sets of exon 2 primers and 1 set of exon 3 primers were designed from the flanking regions of exon 2 and 3 of the canine *Murr1* genomic sequence (**Appendix**). The PCR amplification was conducted by use of 0.15 to 0.25 mg of genomic DNA, 500mM **deoxynucleoside triphosphate (dNTP)**, 0.5mM of each primer, 1X PCR buffer,[a] 1.5mM magnesium chloride, and 0.5 U of *Taq* polymerase.[b] The PCR amplification protocol involved initial denaturation at 94°C for 3 minutes, followed by 30 cycles (94°C for 15 seconds, 60°C for 45 seconds, and 72°C for 1 minute) in an automated thermal cycler.[c] Products of PCR amplification of the C04107 marker were resolved on a 5% gel[d] and developed by end-labeling the reverse primer with ^{32}P and exposing the gel on a film. Other PCR products were resolved on a 1% agarose gel and developed by ethidium bromide.

Southern blot analysis—Restriction enzyme digestion of genomic DNA by use of *Eco*RI and *Bam*HI was performed in accordance with standard protocols.[21] Exon 3 PCR products were used for hybridization after random labeling with [α-^{32}P] **deoxycytosine triphosphate (dCTP)**.[e] Total RNA was extracted from hepatic tissue or blood samples by use of a commercial extraction kit,[f] and **complementary DNA (cDNA)** was obtained with **reverse transcriptase (RT)**-PCR techniques by use of an amplification system.[g] The PCR amplification was conducted, as described previously, by use of 1 mL of cDNA.

Direct sequencing of the *CT-BT* gene—The PCR products were purified by use of a commercial PCR cleaning kit.[h] Products were then sequenced directly by use of capillary electrophoresis[i] with a dye terminator.[j]

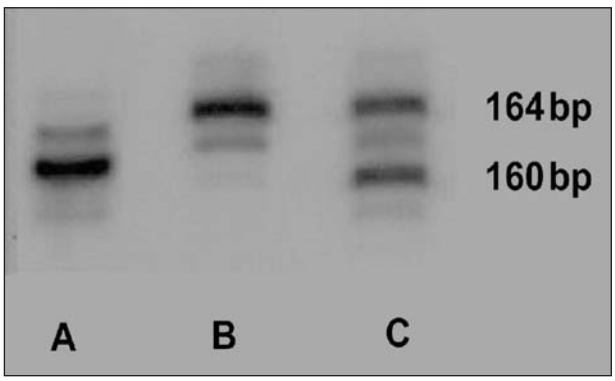

Figure 1—Molecular genetic analysis of representative samples obtained from a cohort of Bedlington Terriers in Australia and analyzed by the C04107 microsatellite marker. Notice the 2 alleles at 160 and 164 bp, respectively. Lanes are as follows: A, dog homozygous for allele 1; B, dog homozygous for allele 2; and C, heterozygous dog.

Results

Conventional diagnostic tests—Twenty-six dogs in the cohort were confirmed to be affected by copper toxicosis on the basis of blood biochemical analysis and evaluation of liver biopsy specimens. Diagnostic criteria for determining the copper toxicosis-affected state were made on the basis of criteria used by other investigators.[22]

Molecular genetic analysis—Two alleles (160 and 164 bp) were identified by use of the C04107 microsatellite marker test (**Figure 1**). Two specific primer sets for exon 2 of *Murr1* were all successfully amplified by use of genomic DNA (**Figure 2**). By use of RT-PCR assay and Southern blot analysis, the exon 2 deletion was verified (**Figure 3**). Two **single nucleotide polymorphisms (SNPs)** were identified in the 5′ flanking region of exon 2, with 1 SNP located at the exon-intron boundary. However, this single base change (adenine for cytosine) in the splice site of exon 2 did not result in any change in the cDNA of *Murr1*, as indicated by results of the RT-PCR assay.

Haplotype analysis—The 131 Bedlington Terriers used in the study represented 8 pedigrees. In this population, 60% were females, and they typically were < 1 year old. Haplotype heterogeneity in this Bedlington Terrier population was found to be 0.74, with the most common haplotypes being H-A and H-B, which accounted for up to 83.1% of the haplotypes in the dogs of the study (**Figure 4**). Haplotype H-C was not detected in our study population. Haplotypes H-C and H-D may have arisen from a

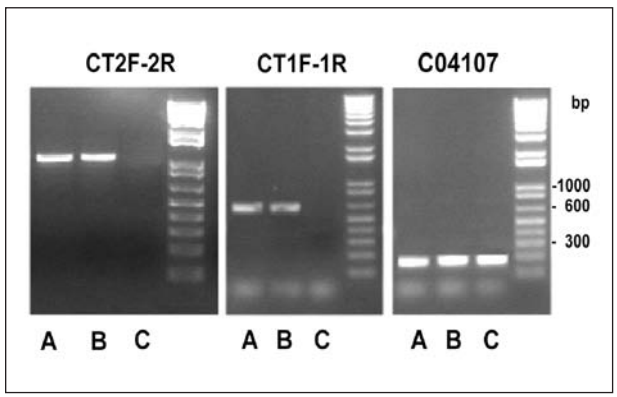

Figure 2—Results of a polymerase chain reaction (PCR) assay for 2 specific primer sets for exon 2 of *Murr1* parallel with C04107 microsatellite marker PCR for verification of successful PCR amplification. Notice the successful amplification of the exon 2 region. Lanes are as follows: A, clinically normal dog; B, dog with heterozygous deletion; and C, dog with homozygous deletion. A 1-kb DNA ladder is on the right side.

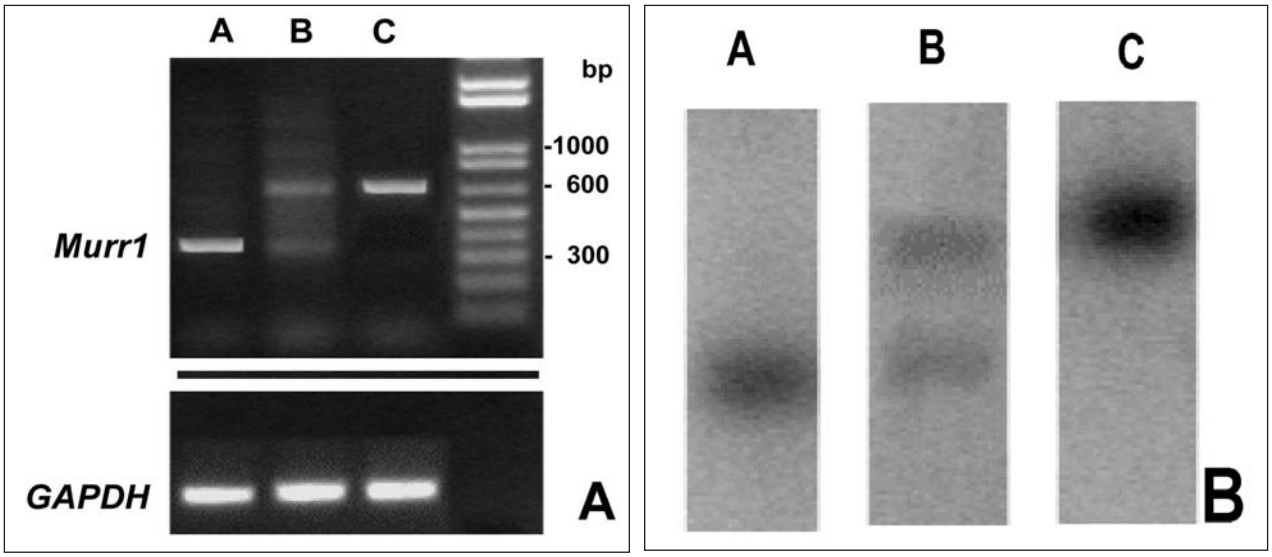

Figure 3—Representative results verifying the exon 2 deletion in samples obtained from Bedlington Terriers and analyzed by use of reverse transcriptase-PCR techniques (A) and Southern blot analysis (B). Lanes are as follows: A, dog with homozygous deletion; B, dog with heterozygous deletion; and C, clinically normal dog. Notice results for the *Murr1* gene and a housekeeping gene (glyceraldehyde-3 phosphate dehydrogenase [GAPDH]). A 1-kb DNA ladder is on the right side of panel A.

recombination event between the C04107 marker and the *CT-BT* gene. Haplotype H-E may have arisen from a transition (cytosine to adenine) in the 5′ splicing site of exon 2 of the *CT-BT* gene. Haplotype H-F may have resulted from a concurrent recombination event with transition (cytosine to adenine).

All phenotypically evaluated homozygous dogs with haplotype H-A were unaffected, whereas homozygous dogs with haplotype H-B had copper toxicosis-affected status. Five heterozygous dogs with haplotypes H-B and H-D had copper toxicosis-affected status, although they had 1 intact exon 2 (**Figure 5**). These dogs had a 2-2 genotype, as determined on the basis of results of the C04107 marker test. In addition, 1 heterozygous dog with haplotypes H-A and H-D was classified as a copper toxicosis-affected status. In this affected dog, both exon 2 regions were intact with a 1-2 genotype, as determined on the basis of results of the C04107 marker test. One homozygous dog with haplotype H-E was suspected of a copper toxicosis-affected status on the basis of annotated history and medical data. This dog had a history of hemolytic diathesis with severe increases in hepatic enzyme activity (alanine transaminase, 159 U/dL; aspartate transaminase, 391 U/dL) and responded to anticupric treatment. The correlation between haplotype and phenotype in several types of heterozygous dogs was unclear.

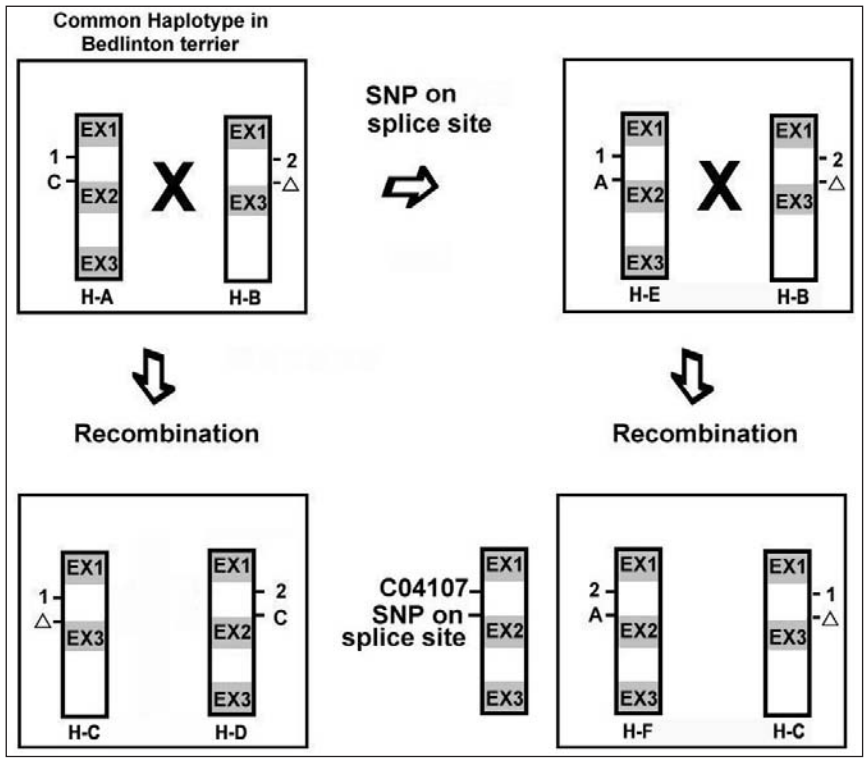

Figure 4—Diagram depicting possible emergence of a novel haplotype for copper toxicosis in Bedlington Terriers. Haplotypes H-A and H-B are the most common haplotypes in Bedlington Terriers. Haplotype H-A is associated with the clinically normal state, and haplotype H-B is associated with the affected state. A recombination event between the C04107 marker and exon 2 of the *Murr1* gene causes the emergence of haplotypes H-C and H-D. The cytosine-to-adenine transition on the splice site is responsible for haplotype H-E, and subsequent recombination may be responsible for haplotype H-F. EX1 = Exon 1. EX2 = Exon 2. EX3 = Exon 3. 1 = Allele 1 of C04107 marker. 2 = Allele 2 of C04107 marker. C = Cytosine on splice site. D = Exon 2 deletion. A = Adenine on splice site. SNP = Single nucleotide polymorphism.

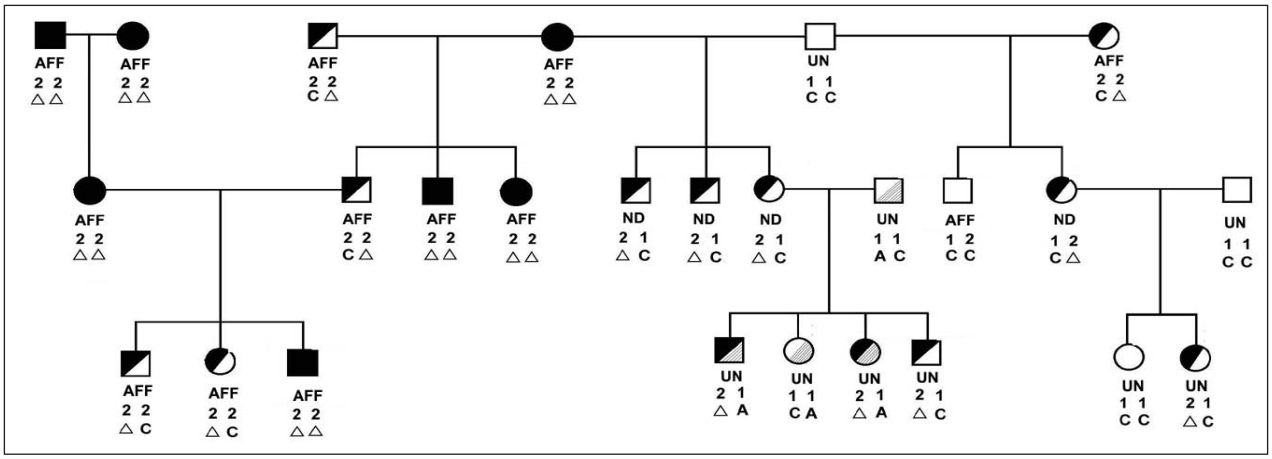

Figure 5—Diagram depicting the pedigree for a family of Bedlington Terriers with a novel haplotype (haplotype H-D). Dogs were considered affected (AFF) when hepatic copper concentration in a biopsy specimen was > 1,000 µg/g of dry weight, and dogs were considered unaffected (UN) when hepatic copper concentration in a biopsy specimen was < 440 µg/g of dry weight. The first row of numbers (ie, 1 and 2) below each dog represents results for the C04107 marker test for each allele. The second row below each dog (ie, D, C, and A) represents results for the SNP on the splice site for each allele. Squares represent male dogs, and circles represent female dogs. Symbols that are solid black represent dogs homozygous for the exon 2 deletion. Symbols that are solid white represent dogs homozygous for exon 2. Symbols that are half black and half white represent dogs that are heterozygous for the exon 2 deletion. Symbols filled with diagonal stripes represent dogs homozygous for a cytosine-to-adenine transition. Symbols that are half white and half diagonal stripes represent dogs heterozygous for a cytosine-to-adenine transition. Symbols that are half black and half diagonal stripes represent dogs heterozygous for the exon 2 deletion with a cytosine-to-adenine transition. ND = Status not determined by examination of hepatic biopsy specimen.

Discussion

The high incidence of copper toxicosis in Bedlington Terriers in Australia may be attributable to the limited number of stud dogs originally imported, which were intensively used for breeding in a relatively small population. Diagnostic tests (eg, hepatic enzyme assays, histologic evaluation for copper by use of special staining techniques, and hepatic determination of copper content) were the conventional methods of choice used to diagnose copper toxicosis in Bedlington Terriers before the introduction of the genetic linkage marker test.[10,23] Results of these diagnostic tests considered separately may not be reliable in providing a definitive diagnosis because these conventional methods can be complicated by several factors that lead to a misdiagnosis for copper toxicosis.

A copper concentration > 400 µg/g of dry weight in dogs > 1 year old, as determined by use of histologic analysis for copper accumulation, was the established standard for diagnosing copper toxicosis in Bedlington Terriers.[10] However, this criterion can be misleading in dogs < 1 year old or dogs fed a diet low in copper because such dogs generally have low hepatic copper concentrations.[17] Typical copper concentrations in clinically normal Bedlington Terriers are < 500 µg/g of dry weight, whereas concentrations in affected Bedlington Terriers are in excess of 800 µg/g of dry weight.[22] However, a conventional diagnosis made on the basis of this criterion may lead to a misdiagnosis and would not identify carrier dogs because copper concentrations in most carrier dogs are < 500 µg/g of dry weight, although the concentrations increase with age.[19,22] Therefore, copper concentrations for clinically normal and affected dogs are difficult to delineate in Bedlington Terriers because affected Bedlington Terriers can have low hepatic copper concentrations, especially when they are young dogs (< 1 year old) or fed a diet low in copper.[19,22] Hepatic copper concentrations in clinically normal Bedlington Terriers are generally higher than concentrations observed in other breeds,[24] possibly because of the breed's association with copper mines[25] and the progressive nature of copper toxicosis, which appears to be an age-dependent phenomenon.

Molecular genetic diagnosis made on the basis of a linkage marker has been widely used to discriminate the carrier state of dogs and reduce the prevalence rate in several countries.[11-14] However, because of recombination events between this linkage marker and the *CT-BT* gene, this linkage marker test has limited diagnostic value.[17] Although it appears to be a reliable test only in families of Bedlington Terriers in which the linkage of the C04107 marker and the *CT-BT* gene is unbroken, many breeders preferentially rely on this marker test for screening of dogs for copper toxicosis.[11-14,17] However, on the basis of our findings, not all affected dogs have the exon 2 deletion in the *CT-BT* gene. Therefore, even though the linkage is unbroken, this marker can still be misleading in families in which the exon 2 deletion is not solely responsible for the pathogenesis.

The gene responsible for copper toxicosis in Bedlington Terriers was identified in the region of CFA10q26, as determined by use of linkage disequilibrium mapping[26] in conjunction with positional cloning of the canine BAC clone.[18] By screening cDNAs closely related to C04107, the *CT-BT* gene (ie, *Murr1*) was identified.[18] Deletion of the entire exon 2 of the *Murr1* gene has been determined by use of RT-PCR and northern blot analysis in samples obtained from affected Bedlington Terriers.[18] Therefore, analysis of results for that study suggested that the detection of the exon 2 deletion in *Murr1* had a predictive value for the screening of dogs for copper toxicosis, at least in their cohort of Bedlington Terriers.[18]

In 1 study,[15] 2 new haplotypes (haplotypes H-E and H-F) were identified in a population of Bedlington Terriers in the United States. These haplotypes may have arisen from a transition (cytosine to adenine) in the 5′ splicing site of exon 2; however, this SNP does not affect the splicing for **messenger RNA (mRNA)**

transcription.[15] Additionally, not all homozygous dogs with allele 2 of the C04107 marker had the homozygous deletion in exon 2, and not all homozygous dogs with allele 1 of the C04107 marker had an intact exon 2 in the *CT-BT* gene.[15] Analysis of these findings indicates that the direct detection of exon 2 and the marker test based on this linkage can also lead to a misdiagnosis in certain populations of Bedlington Terriers, as was evident for the cohort in the study reported here.

Haplotypes H-A and H-B are considered to be the most common haplotypes in Bedlington Terriers. Haplotype H-A is associated with the clinically normal state, and haplotype H-B is associated with the affected state. In addition, haplotypes H-C and H-D are believed to have arisen from a recombination event between the C04107 marker and the *CT-BT* gene, although to our knowledge, haplotype H-C has not been found in any dogs. Finally, haplotypes H-E and H-F appear to have arisen from a cytosine-to-adenine transition in the splicing site of exon 2, with or without a concurrent recombination event. The genetic consequence of this polymorphism is still unclear, but it appears to be associated with copper toxicosis in Bedlington Terriers. In another study,[15] a homozygous dog with haplotype H-E had copper toxicosis, which is consistent with our findings. In that study, 1 heterozygous dog with haplotypes H-B and H-E seemed to be affected by copper toxicosis, as determined on the basis of hepatic copper concentrations, although the rest of the heterozygous dogs were not affected. Those dogs had an exon 2 deletion in 1 allele and a cytosine-to-adenine transition in another allele. If the exon 2 deletion and cytosine-to-adenine transition are both linked to pathogenesis of copper toxicosis in Bedlington Terriers, then all heterozygous dogs with haplotypes H-B and H-E should be affected. This hypothesis questions whether haplotype H-E is truly associated with copper toxicosis in Bedlington Terriers. One possible explanation for this discrepancy may be that the other heterozygous dogs were < 1 year old and fed a diet low in copper, which is often the case. Therefore, those dogs may have been clinically normal when the liver biopsy specimens were obtained. In contrast, the association between haplotype H-E and copper toxicosis in Bedlington Terriers may be the result of a random event and therefore false.

A novel finding in the study reported here was that haplotype H-D was also associated with copper toxicosis in Bedlington Terriers. All dogs that had haplotype H-D (5 heterozygous dogs with haplotypes H-B and H-D and 1 heterozygous dog with haplotypes H-A and H-D) had copper toxicosis. One heterozygous dog with haplotypes H-A and H-D was affected, despite the fact that both its alleles had an intact exon 2. This finding indicated that the deletion of exon 2 in dogs with copper toxicosis was not solely responsible for the phenotype, at least in this cohort of dogs. Furthermore, the cytosine-to-adenine transition did not affect mRNA transcription of the *CT-BT* gene in these dogs. Additional studies are required to determine whether the cytosine-to-adenine transition affects mRNA regulation or is linked to another type of mutation or putative genes located nearby. Therefore, phenotypic predictions made on the basis of results of genetic tests can be complicated for a heterozygous population because multiple factors can be involved (separately or concurrently).

The novel haplotypes found in our study and another study[15] have added to the complexity of the genetics of copper toxicosis in Bedlington Terriers, despite the fact that these findings shed light on the discrepancies between genotype and phenotype in copper toxicosis-affected dogs. This disagreement between results of molecular genetic tests and examination of traditional liver biopsy specimens cannot entirely be attributed to recombination between the marker and the *CT-BT* gene, but it could be attributable to the cytosine-to-adenine transition on the splice site, although the association of this SNP and copper toxicosis is not conclusive.

Analysis of these findings suggests that, in some cases, copper toxicosis in Bedlington Terriers may be caused by another mutation of the *Murr1* gene (affecting regulation of mRNA or a mutation in a promoter region) or mutation of another nearby gene, because our study and another study[15] failed to reveal new mutations that affect the integrity of *Murr1* mRNA. In the case that cytosine-to-adenine transition is not associated with copper toxicosis, it can be explained by locus heterogeneity attributable to differences in copper toxicosis genes located on other chromosomes. Dogs that had a single copy of the exon 2 deletion can represent a haploinsufficient state of the *Murr1* protein that does not severely affect copper excretion. However, if these dogs had another defective gene (another *CT-BT* gene or functionally important gene for copper excretion), they could be affected despite having an intact exon 2 in the gene.

Another possible explanation is the triggering of a compensatory mechanism by turning on a modifier gene or genes, which could compensate for dysfunction of the mutated *Murr1*. Alternatively, the exon 2 deletion may not severely affect the function of *Murr1* or be another isoform of *Murr1*, despite a high homology in mammals.

In a study[27] on function, a human homologue of *Murr1* interacted with the C-terminus of *ATP7B* (Wilson disease gene) but not with *ATP7A* (Menke disease gene) or *Atox1* (copper chaperone gene). This finding explains the reason that human patients with Wilson disease do not have clinical signs indicative of Menke disease despite the high similarity in amino acid sequence between the *ATP7A* and *ATP7B* proteins. Analysis of the results of that study also suggests that *ATP7B* has a specific binding site for *Murr1*, probably at the C-terminus of *ATP7B*, because the interactions were abolished when the C-terminus of *ATP7B* was lacing.[27] In another study[28] on function, it was suggested that *Murr1* is involved in salt homeostasis in epithelium by interaction with the epithelial sodium channel subunit. However, to our knowledge, no study has yet found a functional consequence of the deletion of exon 2 on *Murr1*.

An additional complication is that *Murr1* in humans is unrelated to non-Wilsonian copper toxico-

sis, such as idiopathic copper toxicosis and Indian childhood cirrhosis.[29] These findings have parallels with respect to copper toxicosis in Bedlington Terriers and other breeds of dogs,[3-8] including an emerging new type of copper toxicosis[30] that has differing inheritance modes and pathogenesis. Therefore, genetic defects related to copper toxicosis are complicated and appear to have various genetic causes. Additional studies will be needed to identify the function of other copper-related genes and to clarify the functional consequences of deletion of exon 2 on *Murr1*.

In the study reported here, we found that the genetic diagnosis for copper toxicosis in a population of Bedlington Terriers in Australia can be complicated by haplotype diversity. We also detected a novel haplotype possibly associated with copper toxicosis in Bedlington Terriers.

[a]PCR buffer 3, Applied Biosystems, Foster City, Calif.
[b]Ampli-*Taq* Gold, Applied Biosystems, Foster City, Calif.
[c]PCR sprint, Amplification Technology, Milford, Mass.
[d]Long Ranger gel, BMA, Rockland, Me.
[e][α-^{32}P] Deoxycytosine triphosphate, Geneworks, Melbourne, Australia.
[f]SV RNA extraction kit, Promega, Madison, Wis.
[g]Superscript preamplification system, Invitrogen, Carlsbad, Calif.
[h]PCR Kleen kit, Qiagen, Hilden, Germany.
[i]ABI 3000, Applied Biosystems, Foster City, Calif.
[j]BigDye, version 3.1, Applied Biosystems, Foster City, Calif.

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Appendix is continued on the next page

Appendix

Primers used in molecular genetic analysis of a cohort of Bedlington Terriers in Australia.

Type	Name	Sequence	Size (bp)	Tm (°C)
Exon 2				
	CT1F	5'–TAA GGC TCC ATT GCG ACA G–3'	624	60
	CT1R	5'–TTG CTA ACA TGT AAT GGG AAA CA–3'	624	60
	CT2F	5'–CCT GCA GTT AAG AAG CTG GG–3'	1,054	60
	CT2R	5'–AC CAT GAG GTT GGT TTC CAG–3'	1,054	60
Exon 3				
	EX3-1F	5'–GAA TCA GAA TTT CTG TGT T–3'	107	60
	EX3-1R	5'–CTG GCT GCA TCA GTG TGC TG–3'	107	60
cDNA				
	CTcDNA-F	5'–GCG GGG CTG CTG GCC AGC AT–3'	604	58
	CTcDNA-R	5'–TCA ACA ACT CCA TCT TCA G–3'	604	58

Tm = Primer melting temperature. cDNA = Complementary DNA.