Paper

Population screening for the mutation associated with osteogenesis imperfecta in dachshunds

J. Eckardt, S. Kluth, C. Dierks, U. Philipp, O. Distl

Osteogenesis imperfecta (OI) is a genetic disorder causing defects in the development of collagen type I. Clinical signs of affected dachshunds include multiple fractures of bones, joint hyperlaxity and dentinogenesis imperfecta. Recently, a recessive mutation in the *SERPINH1* gene was detected in dachshunds and enabled the development of a DNA test to identify dachshunds carrying the mutation. The purpose of the present study was to analyse the dachshund breeding population for the frequency of the *SERPINH1* mutation among the nine different breed varieties in dachshunds, birth years and countries of origin. We genotyped the OI-associated *SERPINH1* mutation in 1352 dachshunds from 12 different European countries including all nine varieties. Genotyping was done using a restriction fragment length polymorphism validated by DNA sequence analysis. The overall frequency of OI carriers was 12.9 per cent. Across all different size varieties, the *SERPINH1* mutation was over-represented in wire-haired dachshunds with 17.3 per cent OI carriers. Among the different countries, the proportion of OI carriers was highest in Germany with 20.4 per cent. The test is useful for dachshund breeders to prevent the occurrence of OI-affected dogs and as a diagnostic tool for veterinarians.

Introduction

Osteogenesis imperfecta (OI) is a congenital and inherited disease that is characterised by defects of collagen type I, a major protein component in connective tissue, and of the extracellular matrix of bone. The highly ordered structure of its fibrils stabilises the tissue of bones, teeth, ligaments and sinews. Thus, OI-affected individuals exhibit fragile bones with a high tendency to fracture. In addition, they may also suffer from dentinogenesis imperfecta known as glassy teeth, hearing loss, dwarfism, pulmonary complications and other problems. Several affected patients show blue sclerae (Rau and Glorieux 2004).

In human beings, the majority of cases of OI are caused by dominant mutations in either of two genes encoding for collagen type I, *COL1A1* and *COL1A2* (Marini and others 2007). Additionally, recessive mutations in genes necessary for maturation and correct folding of collagens have been found, which also cause clinical OI: *LEPRE1* (Cabral and others 2007), *PPIB* (Pyott and others 2011) and *CRTAP* (Morello and others 2006). Recent studies discovered that genes of the *SERPIN* family are also associated with human OI, including *SERPINH1* (Christiansen and others 2010) and *SERPINF1* (Becker and others 2011).

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In dogs, OI has been clinically diagnosed in various breeds, including golden retrievers, beagles, collies, poodles, Norwegian elkhounds, Bedlington terriers, and dachshunds (Holmes and Price 1957, Lettow and Dämmrich 1960, Schmidt 1967, Campbell and others 2000, 2001, Drögemüller and others 2009). Canine OI is an autosomal recessive trait (Campbell and others 2000, 2001, Drögemüller and others 2009). Therefore, dogs with clinical signs must have two copies of the mutation, while carriers harbour only one copy and are phenotypically normal but are expected to transmit the mutation with a probability of 50 per cent to their progeny. For the golden retriever, a mutation in *COL1A1* has been identified to be responsible for OI (Campbell and others 2000), whereas a mutation in *COL1A2* causes OI in beagles (Campbell and others 2001). However, collagen genes do not seem to have an influence on the development of OI in dachshunds. Instead, recent studies showed that affected dachshunds carry a recessive T>C mutation in exon 5 of the *SERPINH1* gene (Drögemüller and others 2009) which encodes chaperones responsible for the correct folding of the collagen triple helix (Makareeva and Leikin 2007). In that study, the frequency of OI carriers was 18 per cent in an unrelated population of 113 dachshunds (Drögemüller and others 2009). Apart from this, there is no report about the occurrence and distribution of the mutation within the nine dachshund varieties including wire-, long- and smooth-haired in the different sizes with standard, miniature and rabbit.

Due to the previously reported *SERPINH1* mutation, the development of a genetic test for OI was possible in order to detect carriers within the dachshund breeding population and, thus, to prevent the occurrence of affected dogs in the progeny.

OI-affected dachshunds show typical clinical findings, such as reduced agility, signs of pain when manipulated, brittle and translucent teeth, joint hyperlaxity, spontaneous fractures in long bones and ribs, reduced radio-opacity, but no aberrant haematologic data (Seeliger and others 2003).

The objective of the present study was to determine the frequency of carriers within the dachshund breeding population including all nine varieties of this breed. The distribution by variety, sex, birth year and geographical origin should be analysed to detect a possible prevalence of the mutation. The results of this study will be helpful for breeders and dachshund owners to eliminate this severe disease in puppies, and to prevent a further transmission of the mutation in the breeding population. Veterinarians should become aware of using this DNA test as a diagnostic tool.

Materials and methods

Animals

To obtain samples for this survey, Dachshund breeder clubs were informed about this study and asked to supply DNA samples and pedigrees from their breeding dogs. Breeders from 12 different European countries contributed 1352 samples including 532 EDTA-blood samples taken by veterinarians, and 820 hair root samples taken by breed club supervisors. The majority of samples were sent from Germany with a total number of 1009 individuals, while 127 samples were available from the Czech Republic, 91 samples from France, 37 samples from Italy and 33 samples from The Netherlands. The remaining 49 samples were from Spain, the UK, Belgium, Slovenia, Austria, Switzerland and Luxembourg. For the analysis of the influence of the geographical origin, we only used the five countries with the highest sample numbers, as the others may be insufficiently informative.

The samples covered the whole spectrum of dachshund varieties. All three categories of coat structure, wire-haired (74.6 per cent), smooth-haired (11.8 per cent) and long-haired (13.7 per cent), as well as all three European subcategories of size, standard (64.7 per cent), miniature (22.8 per cent) and rabbit (12.5 per cent), were represented. In our pedigree data which consisted of three generations, we could not detect any cross-breeding of different coat structure varieties.

The number of dogs in each variety was compared with a previous analysis of the German dachshund breeding population. That study found frequencies of 73.7 per cent wire-haired, 20.8 per cent long-haired and 5.5 per cent smooth-haired dogs, as well as 95.7 per cent standard, 4.0 per cent miniature and 1.0 per cent rabbit dachshunds (Gresky and others 2005). Compared with this, the distribution of varieties in our test population roughly matches the general distribution of different sizes and coat structures in the dachshund breed.

All dogs used for this study are current or former breeding dogs. Both sexes were available with 926 female and 426 male dachshunds. The dogs showed a wide distribution of ages with birth years ranging from 1995 to 2012. In order to get a distinguishable overview for our analysis, we formed three groups of birth years. Dachshunds born between 1995 and 2000 were assorted to group 1, dogs born between 2001 and 2007 to group 2 and dachshunds born between 2008 and 2012 to group 3. According to breeding standards, dams may be used for breeding up to an age of eight years, while there are no age restrictions for sires. Thus, birth-year group 1 mainly consists of former breeding dogs, while groups 2 and 3 mainly contain current breeding dogs.

Genotyping of individuals

DNA concentration was measured using the Nanodrop ND-1000 (Peqlab Biotechnology, Erlangen, Germany). As DNA extraction of hair roots is known to sometimes provide insufficient DNA quality, the isolated DNA of the hair root samples was later tested by gelelectrophoresis on a 0.6 per cent agarose gel.

Genomic DNA was extracted from the samples through a standard ethanol fractionation with concentrated sodium chloride (6M NaCl) and SDS (10 per cent SDS).

The DNA isolated from hair roots showed a similarly sufficient quality as the DNA isolated from blood samples and was available in adequate quantity for PCR amplification.

For the screening of the mutation, a PCR was performed. Forward primer 5′-GATGGGTGGTGTGGGTAGAG-3′ and reverse primer 5′-TAGCACCCATGTGTCTCAGG -3′ (Drögemüller and others 2009) were used to amplify a fragment of 451 bp including the mutation. The 20 µl volume reaction contained 10 ng of DNA, 10 pmol of each primer and 0.2 µl *Taq* DNA polymerase (MP Biomedicals Germany, Eschwege, Germany) and started with a four-minute denaturing step at 95°C. This was followed by 38 cycles of 30 seconds at 94°C, 30 seconds at 61°C and 30 seconds at 72°C.

The T>C transition in *SERPINH1* creates a Hpy991 recognition site that was used to perform a restriction fragment length polymorphism (RFLP). This reaction leads to fragments of 336 bp and 115 bp in dogs homozygous for the C-allele and fragments of 451 bp, 336 bp and 115 bp in heterozygous animals. The wild-type T/T is not digested by the enzyme and, thus, only shows the 451 bp fragment (Fig 1). The RFLP reaction was performed using 10 µl of amplicon and 0.3 µl of Hpy991 in a 20 µl reaction mix. The resulting fragments were separated on 2 per cent agarose gels.

To prevent contamination, DNA isolation, PCR and RFLP were performed in different lab rooms, working stations were decontaminated with UV radiation. Sterile plates and filtered pipette tips were used and samples were retested. In order to further confirm RFLP test results, random samples of PCR fragments were sequenced by the automated sequencer Genetic Analyzer 3500 (Applied Biosystems by Life Technologies, Darmstadt, Germany) and used as verified reference samples. In each tested batch, a verified heterozygous and homozygous free control was included. The sequences were analysed using Sequencer 4.8. (Gene Codes, Ann Arbor, Michigan, USA).

The statistical analysis was performed using SAS/Genetics, V.9.3 (Statistical Analysis System Institute, Cary, North Carolina, USA). The procedure ALLELE was used to calculate allele and genotype frequencies. Multiple analysis of variance was employed to test the significance of the phenotypic traits size, coat structure, sex, birth-year cohort and country of origin on the genotype frequency. This analysis was performed using the procedure GENMOD of SAS under a binomial data distribution and a probit link function.

Results

The carrier frequency within the breeding population of 1352 dachshunds amounted to 12.9 per cent. As none of the long-haired dachshunds, and only one of the smooth-haired dogs were detected as carriers of the mutant allele exclusively, the wire-haired dachshund population of 1008 individuals was further considered. In this population, the frequency of carriers was 17.3 per cent. The wire-haired dachshunds screened in this study were progeny of 338 different sires and 407 different dams. In our samples, a total of 420 different kennels were represented (Table 1). The number of samples per kennel ranged from one to 15.

Regarding the size varieties of the wire-haired breeding dogs, carriers reached a frequency of 18.3 per cent in standard dachshunds, followed by rabbit (15.9) and miniature dachshunds (14.4) (Table 2).

In all wire-haired females across all sizes, a frequency of 18.3 per cent was tested to be carriers and 14.8 per cent of all wire-haired male dogs across all sizes were OI carriers.

Among the different countries of Europe, a frequency of 20.4 per cent wire-haired dachshunds from Germany, 10.4 per cent from France, 8.1 per cent from Italy, 5.3 per cent from the Czech Republic and 0.0 per cent from The Netherlands were confirmed to be carriers of the *SERPINH1* mutation (Table 3).

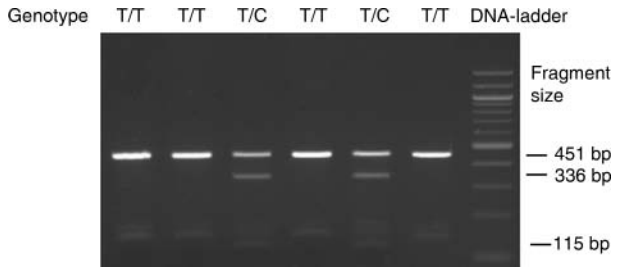

Fig 1: Electrophoretic separation of the canine PCR products for the *SERPINH1* T>C mutation using Hpy991 digestion on a 2 per cent agarose gel. The PCR product of the wild-type allele was not cut (451 bp), while the mutant allele was digested into two fragments (115 bp and 336 bp). In each batch of samples genotyped, a verified heterozygous and homozygous free sample was included

TABLE 1: Number of Different Kennels, Sires and Dams of Wire-Haired Dachshunds Represented by the Samples from the Different Countries

Country	Number of different kennels	Number of different sires	Number of different dams
Germany	295	227	286
France	44	43	44
Czech Republic	34	28	35
Italy	7	7	8
The Netherlands	17	12	14
Other countries	23	21	20
Total	420	338	407

TABLE 2: Results of the Breeding Population Screen for All Nine Dachshunds Varieties Using the Dna-Based Test for the Serpinh1 Mutation

Variety Coat structure	Size	Number of samples	Number of non-carriers (%)	Number of carriers (%)
Wire-hair		1008	834 (82.74)	174 (17.26)
	Standard	707	578 (81.75)	129 (18.25)
	Miniature	194	166 (85.57)	28 (14.43)
	Rabbit	107	90 (84.11)	17 (15.89)
Smooth-hair		159	158 (99.37)	1 (0.63)
	Standard	90	89 (98.89)	1 (1.11)
	Miniature	38	38 (100)	0 (0)
	Rabbit	31	31 (100)	0 (0)
Long-hair		185	185 (100)	0 (0)
	Standard	78	78 (100)	0 (0)
	Miniature	76	76 (100)	0 (0)
	Rabbit	31	31 (100)	0 (0)
Total		1352	1177 (87.06)	175 (12.94)

In wire-haired standard dachshunds, the distribution of the OI carriers by birth years resulted in frequencies of 20.0 per cent in group 1, 16.3 per cent in group 2 and 17.5 per cent in group 3.

Of all phenotypic traits in the analysis of variance, only the carrier frequency among the different coat structure varieties showed a significant association ($P<0.0001$) with the occurrence of the *SERPINH1* mutation. There were neither significant differences among the size varieties across and within coat structure varieties nor among birth-year cohorts in wire-haired dachshunds.

Discussion

The discovery of the T>C mutation of *SERPINH1* associated with OI in dachshunds (Drögemüller and others 2009) enabled the development of a DNA-based test. We used this test for carrier screening within the breeding population of dachshunds from several European countries. Based on a previous analysis of the German dachshund breeding population (Gresky and others 2005), the test population was considered as representative sample for the different dachshund varieties. The high number of different kennels, sires and dams represented by the samples genotyped is indicative for a low level of relatedness between the tested wire-haired dachshunds.

TABLE 3: Results from Samples Submitted to the Screening of the Osteogenesis Imperfecta-Associated Serpinh1 Mutation by Countries for Wire-Haired Dachshunds

Country	Number of samples	Number of non-carriers (%)	Number of carriers (%)
Germany	742	519 (79.65)	151 (20.35)
France	77	69 (89.61)	8 (10.39)
Czech Republic	75	71 (94.67)	4 (5.33)
Italy	37	34 (91.89)	3 (8.11)
The Netherlands	28	28 (100)	0 (0)
Other countries	49	41 (83.67)	8 (16.33)
Total	1008	834 (82.74)	174 (17.26)

Special emphasis was set on the distribution of carriers within all varieties, as well as the correlation between the other phenotypic traits and the presence of OI carriers.

As coat structure is an essential characteristic of domestic dog breeds, we first of all focused on the differences in the varieties of wire-haired, smooth-haired and long-haired dogs. The results showed that the frequency of carriers is significantly higher in wire-haired dogs ($P<0.0001$) than in any other coat variety. In addition to the fact that wire-haired, smooth-haired and long-haired dachshunds are only rarely intercrossed, the high carrier frequency in the wire-haired variety strongly suggests that the OI-associated mutation originated in the wire-haired dachshund population. The single smooth-haired dachshund tested as carrier may be explained by the dominant inheritance of the wire-haired coat structure (Cadieu and others 2009). Therefore, cross-breeding of two heterozygous wire-haired dogs results in 25 per cent smooth-haired offspring. If at least one of the parents was an OI carrier, this may explain the transmission of the mutation to a single smooth-haired dachshund.

In opposition to coat structure, there was no association between the *SERPINH1* mutation and size. Thus, the mutation either arose in dachshunds before the breed was divided into different sizes, or was transmitted to the varying sizes by cross-breeding, which does occasionally occur.

The evaluation of the geographic origin showed a wide distribution of the mutation throughout Europe. Germany had a higher percentage of OI carriers than the other European countries. This may be due to the fact that the dachshund populations of different European countries are based on different founders, and the origin of the *SERPINH1* mutation goes back to breeding dogs from Germany. Therefore, it is likely that the distribution of the mutation has not yet progressed as far in other countries as in Germany. To confirm this, a more evenly spread number of samples from each country would have been necessary, as most of the samples were provided from German breeders.

Considering the birth years, the *SERPINH1* mutation does not seem to be a novel mutation, as it was detectable in every birth-year cohort ranging from 1995 to 2012. The frequency of carriers was similar in all three birth-year cohorts and, thus, constant in former and current breeding dogs. Gender effects are often correlated to genetic disorders. In this case, females are slightly more likely to carry the mutation than males. However, we observed no significant gender differences among the wire-haired varieties.

Regarding all phenotypic traits, only the coat structure is related to the occurrence of carriers. A test to enable breeders of dachshunds to identify carriers, particularly in the wire-haired subpopulation, is a helpful method of selecting against the OI-associated mutation. The testing system used for the present study is a quick and safe way to identify animals carrying the disease-causing mutation. The possibility to avoid matings that harbour the risk of producing affected progeny (carrier x carrier), and also to reduce the dissemination of the OI-causing copy of the *SERPINH1* gene within the dachshund breed is highly beneficial for breeding. Moreover, as carriers appear in almost every European country analysed in our study, it seems to be unavoidable to employ this test in other countries than Germany as well, to prevent a further distribution of the mutation and the occurrence of affected dogs. The latter is an important aspect, since the clinical signs are severe and most of the affected puppies or adolescent dogs are euthanased due to irreparable fractures or poor general condition.

In conclusion, the genetic test is suitable for a secure detection of the *SERPINH1* mutation in dachshunds and, therefore, to optimise breeding strategies and to prevent OI-affected dogs in future progeny. However, considering the high carrier frequencies, carriers should be excluded from breeding slowly but not all at once, as this would lessen genetic diversity considerably. Testing all breeding dogs before their first mating, and mating of carriers only with homozygous-free dogs, will ensure the eradication of OI. Also, the test will help to control the benefit of adapted breeding strategies, to avoid the transmission of the mutation to the future stud dogs, and into the smooth-haired and long-haired dachshund varieties. Moreover, we can approve this test as a non-invasive diagnostic tool to assure clinical signs of OI.

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