

# **Determining whether risk for sebaceous adenitis of Standard Poodles is associated with a specific DLA class II genotype**

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## ***Background information on sebaceous adenitis***

Sebaceous adenitis is an autoimmune disease that is seen mainly in the Standard Poodle, Akita, and English Springer Spaniel. The incidence of the disease in Standard Poodles appears to be low at this time but increasing in frequency over time. The disease is a generalized skin disorder associated with an attack by the dog's own immune system against the sebaceous glands. The breed is known to suffer from two other autoimmune diseases, Addison's disease and autoimmune thrombocytopenia. Addison's disease is due to a similar immune mediated destruction of the adrenal gland and is the subject of other studies here at UC Davis (Dr. Anita Oberbauer, Animal Biology, College of Agriculture). Autoimmune thrombocytopenia is also an autoimmune disease, but the attack is mediated by antibodies rather than immune lymphocytes and the target tissue is the mature platelet in the blood. As far as is known, the three disorders occur independently of each of other.

There are a limited number of published studies on sebaceous adenitis in dogs. The most detailed of these studies was by Hernblad and colleagues (1) in Sweden. A total of 104 cases of SA were studied in English Springer Spaniels (n = 25), Standard Poodles (n = 21) and the Akita (n = 10). These three breeds, together with the Lhasa Apso and the Chow-chow, were the most commonly affected when the incidence was adjusted for the popularity of each breed in the national registry data from the Swedish Board of Agriculture and Swedish Kennel Club. The mean age at diagnosis was 4.8 years. The proportion of males was 61%. Spaniels showed significantly more severe clinical signs than poodles regarding alopecia, seborrhea and secondary bacterial pyoderma. The relationship between otitis externa and pyoderma also differed significantly between the breeds. Euthanasia within 24-48 months was the cause of death in about one-half of the dogs with sebaceous adenitis. Other smaller studies have been reported for the Standard Poodle (2) and Akita (3).

The clinical course of sebaceous adenitis in the Akita was similar to that seen in other breeds including the Poodle (3). The first skin lesions occurred mainly on the midline of the back and on the ears. The age at first onset of the disease was more variable in the Akita than Poodle and the hair loss in Akitas affected mainly the undercoat. The progression of sebaceous gland destruction also varied between dogs and breeds. The disease may end in total destruction of the sebaceous glands, but regeneration of sebaceous glands does occur in many dogs and clinical severity may wax and wane, and not always progress.

Sebaceous adenitis differs from diseases like Addison's, because it becomes clinically apparent long before there is significant destruction of the glandular tissues. Signs of Addison's disease are usually noticed when the adrenal glands have been substantially destroyed and the dog is in a life-threatening Addisonian crisis. At this point immunosuppressive therapy to stop the destruction of the adrenal glands is too late, and therapy must be directed at replacing the mineralocorticoids and glucocorticoids that are no longer being produced.

While a magic bullet for this disease has not been found, most veterinary dermatologists will start off by giving essential fatty acids, certain medicated shampoos, vitamin E, vitamin A, and antibiotics if there is evidence of a secondary bacterial pyoderma. Retinoids will prove effective in some cases but are difficult to obtain. Some dermatologists will be more vigorous in their treatment, especially if the disease is severe and cannot be acceptably controlled by the more conservative approaches. Cyclosporine A with or without prednisolone has been touted in such instances (4, 5). Vigorous treatment can halt the progression of the disease, but signs recur when the therapy is discontinued. Treatment is expensive, and not always totally successful, as judged by the relatively high euthanasia rate for chronically affected dogs (3).

### ***A genetic basis for sebaceous adenitis***

Reichler et al. (3) suggested that sebaceous adenitis is a genetic disorder with an autosomal recessive mode of inheritance. Preliminary whole genome scanning has been done in the UK on 20 SA affected and 25 healthy Standard Poodles using moderate sized single nucleotide polymorphism (SNP) arrays (~20,000 SNPs). This study failed to show an association between any region of the canine genome and disease. However, it is likely that an association can be found by testing several times more affected and non-affected animals and using much larger (~175,000) SNP arrays.

Although whole genome scanning has so far failed to demonstrate a genetic basis for SA, there is strong evidence that autoimmune disorders in both dogs and humans have a strong genetic component. However, the genetics of autoimmune disorders such as SA undoubtedly involve multiple genes in addition to external influences or triggers (epigenetic factors). Indeed, all autoimmune diseases recognized to date in dogs and humans have had one thing in common – they have all been associated with genes that regulate the immune response. These genes are gathered in a distinct region on the dog chromosome 12. An identical region, albeit on different chromosomes, exists in all species of mammals and birds and is known as the major histocompatibility complex (MHC). The MHC of the dog has been specifically designated the “dog leukocyte antigen” (DLA) complex. The MHC (DLA) is subdivided into four regions, containing class I, II, III and IV genes.

A strong association between autoimmunity and the DLA class II genes has been shown for a number of disorders, including Pug Dog Encephalitis (6), a Vogt-Koyanagi-Harada (VKH)-like syndrome of the Akita (7), autoimmune hemolytic anemia, immune arthritis and hypothyroidism in several breeds (8-10), type I diabetes mellitus in

Samoyed dogs, Cairn and Tibetan Terriers (11), anal furunculosis in German Shepherd dogs (12) and a systemic lupus erythematosus-related complex in Nova Scotia Tollers (13). This supports the hypothesis of Svejgaard (14) that many autoimmune disorders of humans, and now dogs, may be MHC associated.

Autoimmune disorders, affecting a wide range of organs and tissues, are becoming increasingly common in pure breeds of dogs (17). The main reason for this increase is inbreeding with a decrease in genetic diversity within the various genes of the DLA complex. This loss of genetic diversity is particularly noticeable in the DLA class II genes, DLA-DRB1, DLA-DQB1 and DLA-DQA1. There are currently 143 known alleles (gene forms) of DRB1, 26 alleles for DQA1, and 66 alleles for DQB1 (Kennedy LJ, personal communication, April 1, 2010). Among all dogs, these various alleles are linked in various three-gene combinations called haplotypes. Over 143 such haplotypes are found in the DLA across all dogs (Kennedy LJ, personal communication, April 1, 2010). However, most purebreds have only a handful of alleles at each of these three gene loci and have as few as 4 to 8 haplotypes in the entire breed (18, 19). The main function of the MHC (DLA in dogs) is what is called self/non-self recognition. The immune system must be able to identify every foreign protein that invades the body, whether it be on a virus, bacteria, fungi, parasite, etc. as being “non-self.” Conversely, the body must be able to recognize every protein that is part of itself and not respond immunologically to it. As genetic variability is lost in the MHC (DLA), the ability to differentiate what is self from what is non-self becomes more and more tenuous. When discernment of self from non-self becomes extremely difficult, the body will start mounting an immune response to its own proteins. The first self proteins that are recognized as foreign are often components of glands (thyroid, sebaceous glands, perianal glands, tear glands, adrenal glands, and parathyroid glands) and proteins on the surface of blood cells (red cells, platelets, white cells). This is probably because proteins on these types of cells more closely resemble the proteins on invading (i.e., foreign) pathogenic organisms.

Given the known association between autoimmune disorders of dogs and specific DLA genetic types (DLA genotypes) (6-13), it seems prudent to determine whether risk for sebaceous adenitis in poodles can be associated with a specific DLA genotype.

Therefore, the first step in identifying a genetic cause for sebaceous adenitis in Standard Poodles would be to sequence certain genes of the DLA complex. If specific alleles or allele haplotypes at certain DLA genes can be associated with a significant risk for sebaceous adenitis, this would be the basis for a genetic test to breed against increased risk for the disease. If a specific DLA genotype cannot be associated with risk for sebaceous adenitis, the search would have to be extended to the entire canine genome, i.e., a whole genome association scan. This would involve the use of very large (>100,000) single nucleotide polymorphism (SNP) arrays, such as those developed by Illumina Corporation. This type of study would be much more expensive.

## **Strategies for achieving the objective described above**

### **1. DLA class II genotyping**

### a. Background

There are two major classes of genes within the MHC (DLA) that control immune responses. The **class I genes** regulate cell mediated immunity. Cell mediated immune response involves a specific class of effector lymphocytes that recognize and destroy cells bearing foreign- (or auto-) antigens. **Class II genes** mediate the production of antibodies. Antibodies recognize and destroy foreign materials and cells by a different mechanism than effector lymphocytes. Therefore, one would expect autoimmune diseases caused by antibodies to be associated with changes in MHC class II genes (e.g., autoimmune thrombocytopenia, autoimmune hemolytic anemia, myasthenia gravis, etc.), while autoimmune diseases caused by immune lymphocytes would be more likely to be associated with changes in MHC (DLA) class I genes (Addison's disease, sebaceous adenitis, anal furunculosis, VKH syndrome, rheumatoid arthritis, thyroiditis, etc). Although this is probably the case, genotyping of DLA class I genes is very difficult and not all of the alleles are known. In contrast, genotyping of the DLA class II genes is very easy and all of the alleles are largely known (15, 16, 18, 19). Sebaceous adenitis involves immune lymphocytes, which would ordinarily require genotyping of the DLA class I genes, which are difficult to type. Fortunately, the genes of the entire MHC (DLA) region are inherited as a block; one block of genes from the sire and one block from the dam. Therefore, it is highly likely that the class I genes of an affected dog will be strongly linked to the class II genes. Therefore, genotyping of the class II genes will provide the same information as genotyping of class I genes. This is why all of the DLA associations with autoimmune diseases in dogs have involved genotyping of type II rather than type I genes (6-13).

### b. Sample collection

We will need DNA-containing specimens from at least 25 dogs suffering from biopsy confirmed sebaceous adenitis and from 50 unaffected dogs, preferably healthy close relatives. *Whole EDTA blood (5 ml in purple topped tubes) is preferred*, because the yield of DNA is much greater than for buccal swabs and the quality is better. A large amount of high quality DNA is not essential for DLA class II genotyping, but would be required if the study were to be later extended to do single nucleotide polymorphisms (SNP) testing using large scale Illumina arrays. Because sample collection from sufficient affected dogs will be the most difficult part of the study, it behooves us to collect as much DNA of high quality as possible in case the study is extended to large scale SNP testing. Many veterinarians will bleed dogs at no cost for such studies, but others will charge for the procedure. Buccal swab kits are much easier and cheaper to collect and would be used for situations where cost of sampling becomes an issue. Buccal swab kits would be sent, upon request, from our Veterinary Genetics Laboratory. Instructions and forms are included separately.

The diagnosis of SA needs to be confirmed in affected dogs. This requires several small skin biopsies taken from the margins of healthy and diseased tissues. These are fixed in formalin and usually sent to a commercial veterinary pathology service, where they are studied under the microscope and a diagnosis made. This will involve the cost of taking

the biopsy and the cost of having it examined by a certified veterinary pathologist. If expense of either becomes an issue, there are two alternatives. The dog can be seen at our veterinary medical teaching hospital dermatology service at no cost and biopsies examined by our pathologists. If the dog is not close to our veterinary hospital, the biopsies can be taken by the local veterinarian and sent to us for free examination by one of our pathologists.

#### c. Procedure for typing class II genes

The DLA class II genes, DRB1, DQA1, and DQB1, will be typed by DNA sequence analysis with locus-specific intronic primers. The 270-bp polymorphic sequence in exon 2 of the DLA-DRB1 was amplified using primers “DRBF” (5'-gatccccccgccccacag-3') and “DRBR” (5'-tgtgtcacacacctcagcacca-3'). The 307-bp polymorphic sequence in exon 2 of DLA-DQA1 was amplified using primers “DQAF” (5'-taaggttctttctccctct-3') and “DQAR” (5'-ggacagattcagtgaagaga-3'). The 267-bp polymorphic sequence in exon 2 of the DLA-DQB1 was amplified using primers “DQBF” (5'-tcactggccccggctgtctcc-3') and “DQBR” (5'-ggtgctgctcacctcgccgct-3'). PCR was carried out in 20 µl reaction volumes containing 50ng DNA, 0.2 mM dNTP, 0.25 µM forward and reverse primers in 1 × PCR buffer, and 1 unit HotStarTaq Plus® polymerase (QIAGEN; Germantown, MD). The mixture was then incubated for 5 min at 93°C followed by 30 cycles of 45s at 93°C, 45s at 61°C for DRB1, 57°C for DQA1 and 66°C for DQB1, and 1 min at 68°, with a final extension of 10 min at 68°C. The PCR products were separated by electrophoresis on 0.8% agarose and purified using a QIAquick gel purification kit (QIAGEN). The DNA was eluted with 50 µl elution buffer.

Sequencing of exon 2 regions of the DLA-DRB, -DQA and -DQB genes was performed on both strands for the NME affected dog samples, and on one strand for unaffected dog samples. For one direction sequencing, the reverse primers for DRB1 and DQB1 and forward primer for DQA1 were used. The purified PCR products were sequenced with a Big Dye® Terminator (Applied Biosystems) in a 15 µl reaction containing 1 µl Big Dye® terminator mix, 2 µl reaction buffer (5×), 35 ng sequencing primer, and 3 µl purified PCR product. The sequencing reaction was incubated at 93°C for 2 min and amplified for 40 cycles at 93°C for 20 s, 50°C for 20 s, and 60°C for 4 min. Unincorporated dye terminators and dNTPs were removed by gel filtration (Performa DTR Ultra; EdgeBio, USA) and the amplified products were analyzed by capillary electrophoresis using an ABI 3730 Genetic Analyser (Applied Biosystems). Vector NTI advance™ software (Invitrogen; Carlsbad, CA) was used for alignment of sequence data.

#### d. Haplotype assignment and statistical analyses

Allele nomenclature will be determined from known sequences in Genbank and in the Immune Polymorphism Database (<http://www.ebi.ac.uk/ipd>). Haplotype combinations of

DLA-DRB1/DQA1/DQB1 will be determined for all dogs by the method of Kennedy et al. (15, 16).

We anticipate that certain alleles at each of these three DLA class II genes will occur much more frequently in affected than non-affected dogs. The significance of certain DLA-DRB1, -DQA1 and -DQB1 alleles to sebaceous adenitis will be determined using odds ratios (OR). If there is no difference in the incidence of certain alleles in affected and non-affected dogs, the OR will be 1.0. If certain alleles occur more often in affected than non-affected dogs, the OR will be greater than 1.0. The higher the OR is, the stronger the genetic association and the greater the risk of developing sebaceous adenitis. For instance an OR of 10 indicates that a dog with a certain DLA class II genotype would be 10 times more likely to develop sebaceous adenitis than a dog with an OR of 1.0 or less than 1.0. As has been noted in most autoimmune disorders in dogs, there are additional factors that increase risk. The first is a linkage between alleles of one class II gene and alleles of the other two genes (6, 7, 12). These are known as haplotypes. A haplotype is a combination of alleles at different genes that always occur together. A second factor that increases risk is homozygosity (6, 7, 12). Homozygosity is when both alleles at the same gene are identical. Homozygosity is greatly increased by inbreeding often extends to haplotype as well as single genes. Therefore, most autoimmune diseases of dogs are associated with specific alleles at each DLA class II gene, specific haplotypes across the three genes, and increased homozygosity at each gene loci. The significance of any given OR can be determined by measuring confidence intervals.

e. Timing of project - The time required to do this study is based almost entirely on the time it takes to obtain sufficient numbers of DNA- containing samples from affected dogs. Obtaining samples from normal dogs will be relatively easy and rapid. The time it takes to get sufficient samples from affected dogs will depend both on the incidence of sebaceous adenitis in Standard Poodles, and the willingness of owners and breeders to contribute samples. If sufficient samples were in hand, the actual DLA class II genotyping could be done within weeks. I would anticipate that this first stage of studies could be done in one year. More in depth studies, if required, would be logical extensions of the current study and involve the same samples. These additional studies would require much more financial support and a longer study period.

## 2. Whole genome scanning

a. Background - Whole genome scanning involve the use of large panels (~75,000 – 200,000) of genetic markers equally spaced across every chromosome of the dog. The genetic marker of choice is the SNP (single nucleotide polymorphism). Individual dogs differ from each other at hundreds of thousands of points (nucleotide sequences) across their genome. Some of these differences are random, while others are associated with the various traits that determine an animal's appearance, behavior and propensity for disease. The goal is to identify one or more specific types of SNPs that occur only in the affected dogs. If one is lucky, this difference may be actually associated with the

causative gene or genes. More commonly, the associated SNP will not involve the causative gene itself, but rather will be at another gene within the same region of the chromosome.

b. Sample collection – The same samples used for DLA class II genotyping will be used for whole genome scanning.

c. Fine mapping and candidate gene mapping - Once you identify places in the genome where strong associations exist in diseased animals, you can then do what is called fine mapping or candidate gene mapping. Fine mapping requires a careful search of all of the genes in the region of the association with the goal of finding the actual abnormal gene responsible for the disease in question. Candidate gene mapping can narrow the search from dozens of genes in the region of the association to a single gene. Candidate genes are genes that are known to cause a similar or identical disease in humans or other animals. Good candidate genes for SA would be genes that have been found to be associated with autoimmunity in people.

d. Collaborations – Whole genome mapping will be done in collaboration with groups already involved with this type of study on SA in Standard Poodles. In particular, the group at the Animal Health Trust in Newmarket, UK headed by Mike Bournnell PhD.

e. Timing of project – We intend to work with Dr. Bournnell and his group by providing them with the extra samples that they need to complete their whole genome scan project. In turn, we will hopefully be able to access some of their samples for our studies on the DLA class II genes. Whole genome scanning is relatively fast once the appropriate samples are obtained (weeks). However, homing in on the exact causative gene can take a much longer time (months, years).

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