

# Association of Doberman hepatitis to canine major histocompatibility complex II

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## Abstract

Doberman hepatitis (DH) is a chronic and progressive inflammatory liver disease that mainly affects female dogs. The high incidence of chronic hepatitis in Dobermans is suggestive of a genetic predisposition. DH is characterized by mononuclear cell infiltration and copper accumulation in the liver and major histocompatibility complex (MHC) class II antigen expression in the hepatocytes. In dogs, the MHC is referred to as the dog leukocyte antigen (DLA) system. In this study, the potential role of *DLA* genes in DH was investigated by sequence-based typing in the exon 2 of *DLA-DRB1*, *-DQA1* and *-DQB1*. The case group comprised 37 Dobermans with subclinical or clinical DH. The control group consisted of 37 healthy Dobermans, with normal liver enzyme values and without immunosuppressive medication. The control dogs were over 10 years old to include dogs with the lowest genetic risk of DH. Our results indicate that Dobermans with homozygous *DLA-DRB1\*00601/DQA1\*00401/DQB1\*01303* [odds ratio (OR) = 14.9, confidence limit (CL) = 3.1–71.7,  $P < 0.00005$ ], especially with homozygosity for *DLA-DRB1\*00601* ( $P < 0.0005$ ), are susceptible to DH. The DQ heterodimer *DLA-DQA1\*00901/DQB1\*00101* and the allele *DLA-DRB1\*01501* appear to confer protection against DH ( $P < 0.001$ ). Allele and haplotype frequencies were compared using chi-squared statistics. The disease shows a complex pattern of inheritance, but the observed DLA class II association with DH suggests a role for the immune system in the development of the disease.

## Introduction

Doberman hepatitis (DH) is a chronic and progressive inflammatory liver disease first recognized in the early 1980s (1). The disease primarily affects female dogs (2–6) and is most frequently seen in middle-aged Dobermans, although the age of onset can vary. DH occurs in subclinical and clinical forms. The disease differs clearly from other forms of hepatitis in other breeds. It tends to be more aggressive, and the prognosis of this condition is poor if associated with clinical signs of liver failure. Despite treatment with corticosteroids, the disease is fatal (3, 5, 7, 8). The best way of identifying dogs affected by subclinical disease is routine laboratory assessment of liver-associated variables from blood

specimens. An increase in the alanine aminotransferase (ALT) serum level is detected before an increase in the alkaline phosphatase (AP) (9). For definitive diagnosis of DH, a liver biopsy is recommended after obtaining several ALT readings that are at least threefold above the upper reference limit (10).

The high incidence of chronic hepatitis in Dobermans is strongly suggestive of a genetic predisposition (2, 3, 5, 9, 11). The etiology and pathogenesis of DH remain poorly understood, but two theories have been formulated. According to the first, DH is believed to be a form of copper toxicosis. Dobermans with subclinical hepatitis associated with increased copper concentrations have been suggested to have a reduced biliary excretion of copper caused by an abnormal copper metabolism (12, 13). According to the second theory, DH is immune-mediated in origin, and a T-cell mediated autoimmune response is activated in

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genetically predisposed individuals (9). Affected hepatocytes express major histocompatibility complex (MHC) class II antigens, which suggests that DH is an autoimmune disease (14). Although viral antigens have been shown to induce aberrant expression of MHC class II on epithelial cells (15), the inheritance rates of DH argue against the latter theory.

MHC class II genes encode the products that play a key role in the maintenance of homeostasis and tolerance of the immune system. MHC class II antigens mainly determine which antigenic peptides an individual is able to present to CD4+ T-lymphocytes in order to stimulate an immune response (16). In humans, expression of MHC class II is normally restricted to professional antigen-presenting cells, that is, dendritic cells, macrophages, B-cells and thymic epithelial cells, but it can be induced in other cell types by inflammatory conditions in the target organs of autoimmune diseases (17–19). MHC class II genes are expressed either constitutively or upon stimulation by cytokines (20–22). In human liver disorders, MHC class II expression is seen in hepatocytes in primary biliary cirrhosis and chronic sclerosing cholangitis, both regarded as autoimmune liver diseases (23, 24). The density of class II molecules on the cell surface also plays a crucial role in antigen presentation (25). In DH, MHC class II expression was shown to be persistent and increased on the hepatocyte membrane during disease progression (14). When MHC class II antigens are not expressed on cell membranes, the cells have been suggested to escape immune attack mediated by CD4+ T-cells. When the MHC class II molecule reaches the cell surface with its antigen peptide bound to it, the hepatocyte acts as an antigen-presenting cell and initiates an immune response, leading to destruction of liver cells (14).

The canine MHC, also termed the dog leukocyte antigen (DLA) gene system, has been shown to be highly polymorphic and is well characterized (26). This gene region has been extensively studied in recent years. The role of DLA in disease susceptibility has been established for a range of immune-mediated diseases, including canine rheumatoid arthritis (27), diabetes (28), lymphocytic thyroiditis (29, 30) and immune-mediated hemolytic anemia (31). Lymphocytic thyroiditis is frequently seen in the Doberman breed (29, 30). The occurrence of common features of autoimmune diseases and the co-association of multiple autoimmune diseases in the same individual or family support the notion that common genetic factors may predispose to autoimmunity (32, 33).

The aim of our study was to investigate the relationship between DLA class II antigens and DH. We found a strong association of the homozygous haplotype DLA-DRB1\*00601/DQA1\*00401/DQB1\*01303, especially the homozygous allele DLA-DRB1\*00601, with disease in the affected dogs. In addition, the DLA-DQ heterodimer DLA-DQA1\*00901/DQB1\*00101 and allele DLA-DRB1\*01501 may be protective against DH. The background of the disease is unknown, but the observed DLA class II association with DH suggests an immune nature of the disease.

## Material and methods

### Study material

Eighty-six privately owned Finnish Dobermans were examined in this study with their owners' consents. The case group comprised 37 Dobermans with subclinical or clinical hepatitis. Twenty subclinical dogs had been followed for months, showing consecutive ALT readings at least threefold the upper reference limit (18–77 U/l). Seventeen clinical cases manifested typical hepatitis symptoms. Diagnosis of hepatitis was confirmed histologically in both groups with hematoxylin and eosin stains and increased copper contents with rubeinic acid stains. For DNA, liver samples were taken postmortem from dogs with clinical signs of chronic hepatitis. Eighteen liver samples and two ethylenediaminetetraacetic acid (EDTA) blood samples were collected from dogs with subclinical hepatitis. The liver samples were collected during 1981–2008 and stored at –20°C. For the control group, we collected EDTA blood samples from dogs that had no signs of liver failure or immunosuppressive medication. The control dogs were over 10 years old so as to include only dogs with the lowest genetic risk of DH. In the literature, the youngest Doberman reported to suffer from DH was aged 1.5 years, and the oldest was 11 years (3, 11). Serum ALT levels were measured in all control dogs to ensure that they were within the normal range (18–77 U/l). In total, 49 blood samples were collected and 37 control dogs were included in the study. The remaining 12 dogs were excluded because of abnormal ALT readings. The pattern of inheritance was studied with pedigree analysis to make sure that the dogs were not closely related. Sixty-six out of the 74 dogs analyzed in total were unrelated at least to the grandparental level. However, three unrelated dogs were found as grandfathers in the case group: dogs A and B had three descendants, and dog C had two descendants at the grandparental level. All these cases had different parents.

### DNA isolation, PCR and sequencing

DNA was extracted by using either the QIAamp® DNA Mini Kit for tissues (Qiagen, Valencia, CA) or the Puregene® DNA kit for EDTA samples (Gentra Systems Inc., Minneapolis, MN). The DNA concentrations were measured with a NanoDrop ND-1000 UV/Vis spectrophotometer (NanoDrop Technologies, Inc. Wilmington DE USA), and the samples were adjusted to 10 ng/μl for PCR. Specific DLA-DRB, -DQA, -DQB- and M13-tailed primers (Table 1) were used to amplify and sequence the DLA loci, and the final product sizes were 356, 329 and 334 bp. All polymerase chain reactions (PCRs) were performed in a 20-μl reaction containing 1× PCR buffer II (Applied Biosystems, Foster City, CA), 2.5 mM of MgCl<sub>2</sub>, 0.5 μM of each primer, 0.2 mM of dNTP, 0.15 U of AmpliTaq Gold™ (ABI PRISM® BigDye™ Terminator Cycle Sequencing Ready Reaction Kits, Original

**Table 1** Primers used to amplify and sequence the *DLA-DRB1*, *-DQA1* and *-DQB1* genes

Primer name	Primer sequence	References
DRB1_F	gtt ttc cca gtc acg acc cgt ccc cac agc aca ttt c	34
DRB1_R	cag gaa aca gct atg act gtg tca cac acc tca gca cca	34
DQA1_F	gtt ttc cca gtc acg acc tca gct gac cat gtt gc	35
DQA1_R	cag gaa aca gct atg acg gac aga ttc agt gaa gag a	36
DQB1_F	gtt ttc cca gtc acg acc tca ctg gcc cgg ctg tct c	36
DQB1_R	cag gaa aca gct atg acc acc tcg ccg ctg caa cgt g-	36
M13_F	gtt ttc cca gtc acg ac	36
M13_R	cag gaa aca gct atg ac	36

DLA, dog leukocyte antigen.

and Version 2.0 Protocol, Applied Biosystems) and 40 ng of genomic DNA. The PCR program had an initial denaturation at 95°C for 5 min, followed by 35 cycles at 95°C for 30 s, 57°C for 30 s and 72°C for 45 s. The final extension was made at 72°C for 10 min. PCR products were purified by EXOsap-IT (USB Corporation, Cleveland, Ohio, USA) before sequencing. DNA sequencing was performed with an ABI 3730xl DNA Analyzer (Applied Biosystems).

### DLA haplotype assignment

DLA alleles were identified by sequence-based typing, and the sequences were compared with an existing consensus sequence in the MatchToolsNavigator program (Applied Biosystems). The corrected sequence was evaluated against a reference sequence library (<http://www.ebi.ac.uk/ipd/mhc/index.html>) using MatchTools program (Applied Biosystems) to obtain the alleles. Previous knowledge of DLA allele combinations in Dobermans was used to facilitate the haplotype construction. Allele and haplotype frequencies were compared using chi-squared statistics. Odds ratios (ORs) and the relative risk with 95% confidence limits (CLs) were calculated.

### Results

Our study material included 74 Dobermans: 37 DH cases and 37 healthy controls. There were approximately three

**Table 2** DLA haplotype frequencies in Dobermans with DH and in healthy controls

DRB1	DQA1	DQB1	Cases (n = 37)		Controls (n = 37)	
			Number	Frequency (%)	Number	Frequency (%)
00601	00401	01303	35	94.6	20	54.1
00601	005011	00701	2	5.4	1	2.7
01501	00901	00101	—	—	12	32.4
01502	00601	02301	—	—	1	2.7
028v	00401	01303	—	—	1	2.7
00201	00901	00101	—	—	1	2.7
00101	00101	03601	—	—	1	2.7

DH, Doberman hepatitis; DLA, dog leukocyte antigen.

times more affected females than males (75.7% vs 24.3%,  $P = 0.0056$ ) in our case group; in the control group, these proportions did not differ significantly (54.1% vs 45.9%). We found a total of seven different DLA class II haplotypes (Table 2), and in the control group males had more variation in different haplotypes than females (6 vs 3). The two most common haplotypes (haplotypes 1 and 3) were found in 95% of cases and controls. Haplotype 1 was carried by the majority of females (27 cases and 14 controls), while only 14 males had this haplotype present (8 cases and 6 controls), and of all the 48 females in this study, 85.4%, carried this haplotype. Haplotype 3 was seen only in the control group in seven males and five females. One male control dog carried DLA-DRB1\*028v/DQA1\*00401/DQB1\*01303 (haplotype 5), a previously unpublished haplotype for Dobermans (Table 2).

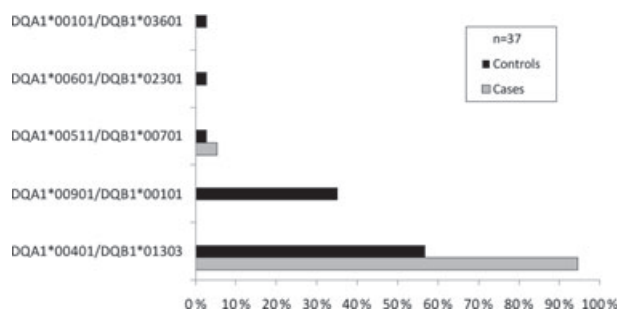
Of the seven haplotypes, the DH cases carried only two of them, haplotypes 1 and 2 (Table 2). All DH cases, but only 56.8% of controls, were homozygous for DLA-DRB1\*00601 ( $P < 0.0005$ ) (Table 2). Of the affected dogs, 94.6% were homozygous for haplotype 1, DLA-DRB1\*00601/DQA1\*00401/DQB1\*01303. This haplotype was found to be homozygous in only 54% of control Dobermans (Table 3). All dogs, both cases and controls, carried at least one copy of the haplotype DLA-DRB1\*00601/DQA1\*00401/DQB1\*01303, but we observed a highly significant association of DH with homozygosity of this haplotype [ $P < 0.00005$ , OR = 14.9, 95% CL = 3.1–71.7]. The overall heterozygosity of DH dogs was significantly reduced compared with controls (5.40% vs 45.95%,  $P < 0.001$ , Table 3).

In addition, we found putative protective alleles in the control group. One particular DQ heterodimer, DLA-DQA1\*009

**Table 3** Frequencies of overall DLA-DRB1\*00601/DQA1\*00401/DQB1\*01303 haplotype homozygosity and heterozygosity in DH cases and controls

	Cases (n = 37)		Controls (n = 37)		$\chi^2$	OR	95% CL	P value
	Number	Frequency (%)	Number	Frequency (%)				
Homozygous	35	94.6	20	54.1	18.7	14.9	3.1–71.7	<0.00005
Heterozygous	2	5.4	17	45.9	—	—	—	—

DH, Doberman hepatitis; DLA, dog leukocyte antigen.

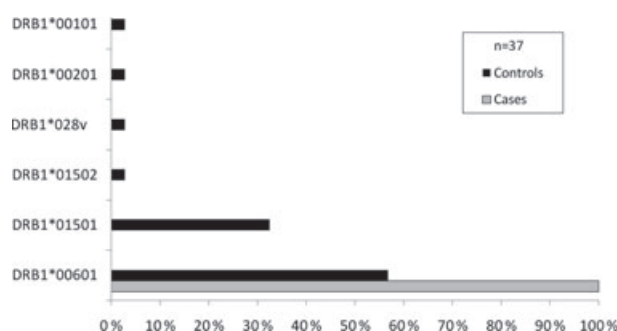


**Figure 1** Frequencies of dog leukocyte antigen (DLA)-DQ heterodimer alleles in DH cases and controls. DLA-DQA1\*00901/DQB1\*00101 heterodimer was present only in the control group ( $\chi^2 = 15.8$ ,  $P < 0.001$ ).

01/DQB1\*00101, was present in 35% of healthy Dobermans ( $P < 0.001$ ), but was not observed in any of the DH cases (Figure 1). DLA-DRB1\*01501 was in linkage disequilibrium with DLA-DQA1\*00901/DQB1\*00101 (Table 2). This allele was found to be present in 32.4% of the controls and did not appear among DH cases (Figure 2).

## Discussion

This study has, for the first time, shown a significant association between DH and DLA class II. A strong association of the homozygous haplotype DLA-DRB1\*00601/DQA1\*00401/DQB1\*01303 with the presence of DH was observed (OR = 14.9, CL = 3.1–71.7,  $P < 0.00005$ ). Homozygosity for DLA-DRB1\*00601 shows strong linkage to the disease ( $P < 0.00005$ ). Surprisingly, 100% of the DH cases were homozygous for DLA-DRB1\*00601, while this allele was homozygous in only 56.8% of the controls. In addition, 94.6% of our DH cases carried homozygous haplotype DLA-DRB1\*00601/DQA1\*00401/DQB1\*01303. This haplotype was very common in Dobermans in a previous study dealing with hypothyroidism in connection with DLA class II, being homozygous in 49.6% and heterozygous in 50.4% of the 135 Dobermans investigated (30). We found a similar ratio



**Figure 2** Frequencies of dog leukocyte antigen (DLA)-DRB alleles in DH cases and controls. DLA-DRB1\*01501 was present only in the control group ( $\chi^2 = 14.3$ ,  $P < 0.001$ ).

in our control group, which consisted of elderly Dobermans with a low genetic risk of contracting DH. All dogs, both cases and controls, carried the characteristic ‘Doberman haplotype’ DLA-DRB1\*00601/DQA1\*00401/DQB1\*01303, but when homozygous, it increased the odds of getting DH almost 15-fold. The haplotype DLA-DRB1\*00601/DQA1\*00401/DQB1\*01303 is rare in the general dog population, to date found only in Dobermans, Rough Collies and mongrels from Brazil. This result suggests that the homozygous haplotype DLA-DRB1\*00601/DQA1\*00401/DQB1\*01303, and especially DLA-DRB1\*00601, is associated with susceptibility to DH.

In humans, homozygosity for certain HLA class II alleles has been shown to be associated with disease susceptibility to such autoimmune diseases as celiac disease and autoimmune hepatitis type-1 (37). Being homozygous for certain HLA class II alleles has also been shown to affect the severity of disease outcome in multiple sclerosis (38), celiac disease (39) and in rheumatoid arthritis (40). Different dog breeds have been developed as a result of aggressive phenotypic selection, in many cases resulting in inbreeding and a lack of genetic diversity. High interbreed variation and low intrabreed variation have been shown to be associated with susceptibility to infections and autoimmune diseases in dogs (41). Variations in MHC class II allele and haplotype frequencies may explain why individual dogs or certain breeds are good or poor responders to particular microbial antigens or why certain dogs are prone to autoimmunity and the development of autoantibodies (42). The function of MHC class II molecules is to present antigens to cells of the immune system, and dogs with limited diversity clearly respond to a more narrow range of antigens. Because of the restricted range of mounted immune responses, homozygous dogs can be more susceptible to microbial or autoimmune diseases. The DLA region showed an extremely limited variation in the DH group, with only two different haplotype combinations compared with seven in the control group.

In contrast, a relatively rare heterodimer in this breed, DLA-DQA1\*00901/DQB1\*00101, was found to be overrepresented among controls (35%) and had a highly significant negative correlation with DH ( $P < 0.001$ ). This allele combination was completely absent in the patient group. A similar negative correlation with DH was observed for the DLA-DRB1\*01501 allele. This particular allele was identified in 32.4% of controls and did not appear among DH cases. DRB1\*01501 was in linkage disequilibrium with the heterodimer DLA-DQA1\*00901/DQB1\*00101 (Table 2). DLA molecules are heterodimeric cell surface glycoproteins, consisting of two protein chains, called  $\alpha$  and  $\beta$ . The  $\alpha$  chains are encoded by *DLA-DQA1* and *DLA-DRA1* genes, and the  $\beta$  chains by *DLA-DQB1* and *DLA-DRB1* genes. The DR $\alpha$  locus appears to be functionally monomorphic in dogs (43). Thus, the allele DLA-DRB1\*01501 makes an important contribution to the DR heterodimer structure. The DLA heterodimers

are otherwise highly polymorphic. This genetic variation is essential for the immune system because the DLA antigens function as antigen-presenting molecules for both foreign and endogenous antigens. Genotype-specific differences in tolerance development could be the basis for associations between DLA genes and autoimmune disease. In humans, HLA-DQ molecules have been proposed to be more closely involved in repertoire selection in the thymus, while HLA-DR molecules may be more involved in peripheral antigen presentation (44). The regulation of genes required for MHC class II-restricted antigen presentation plays a fundamental role in the adaptive immune response because the encoded molecules direct the development, activation and homeostasis of CD4<sup>+</sup> T-cells (45). In humans, HLA-DQ molecules have been recognized to be both mediators of several autoimmune diseases and also involved in protection (46). In dogs, the DLA-DQA1\*004/DQB1\*013 heterodimer appears to confer protection against diabetes (28).

The human MHC class II antigen region on chromosome 6 has been associated with nearly all autoimmune diseases (47). For complex disorders, an HLA association can increase or decrease the risk of disease and dosage effects may occur (37). One theory for the association between MHC genotype and susceptibility to an autoimmune disease is based on the differences in the ability of different allelic variants of MHC class II molecules to present autoantigenic peptides to autoreactive T-cells. An alternative hypothesis for the association proposes that MHC alleles shape the T-cell repertoire. According to this theory, self-peptides may drive the positive selection of developing thymocytes that are specific for particular autoantigens (25). Increased endothelial MHC class II expression has been shown in a number of autoimmune diseases, including rheumatoid arthritis, systemic lupus erythematosus, multiple sclerosis and Crohn's disease (49). The importance of hepatocytes presenting MHC class II antigens in connection with DH is unknown, but an immune response is clearly maintained and modulated through the interaction between immune cells and cells of the target organ. The level of MHC class II expression was also found previously to correlate with the severity of DH (14).

Our results indicate that Dobermans with the homozygous, high-risk haplotype DLA-DRB1\*00601/DQA1\*00401/DQB1\*01303, especially with homozygosity for DLA-DRB1\*00601, are susceptible to DH. The DQ heterodimer DLA-DQA1\*00901/DQB1\*00101 and the allele DLA-DRB1\*01501 appear to confer protection against DH. The disease shows a complex pattern of inheritance, but our findings indicate a strong association between DH and DLA class II. Our results therefore suggest an immune origin of DH. As in human autoimmune disorders, it is likely that MHC class II genes contribute only a proportion of the total genetic susceptibility to the disease, and further work is required to identify other susceptibility genes as well as other immunodiagnostic features.

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