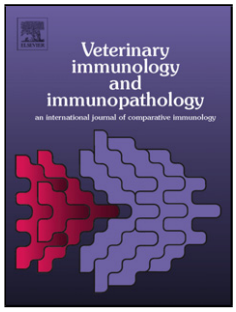

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Single Nucleotide Polymorphisms in Major histocompatibility class II haplotypes are associated with potential resistance to inflammatory bowel disease in German shepherd dogs.

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Highlights

This study has identified an association between DLA-type and canine IBD.

- The first protective (DLA-DRB1*015:02/DQA1*006:01/DQB1*023:01) DLA haplotypes for canine IBD in GSD was identified.
- Protective haplotype was found only in the control population and was associated with a reduced risk of IBD ($P<0.001$).
- Also, an association with IBD was identified for a haplotype common to GSDs (DLA-DRB1*015:01/DQA1*006:01/DQB1*003:01).
- The haplotype DLA-DQA1*015:01/DQA1*006:01/DQB1*003:01 was more prevalent in IBD dogs than controls (31.25% of the cases compared with 18.18% of the controls, OR=1.93, CI= 1.02_3.67, P=0.05), indicating that it may be a risk factor for IBD.

Abstract

German shepherd dogs (GSD) in the UK are at increased risk of developing the Inflammatory Bowel Disaese (IBD). IBD is believed to be a multifactorial immune mediated disease affecting genetically predisposed dogs. The aim of the current study was to investigate whether susceptibility to IBD in GSD is associated with the major histocompatibility complex (MHC) class II locus (Dog Leukocyte Antigen, DLA). Sequence-based genotyping of the three polymorphic DLA genes DLA-DRB1, -DQA1 and –DQB1 was performed in 56 GSDs affected by IBD and in 50 breed-matched controls without any history of gastrointestinal signs. The haplotype DLA-DRB1*015:02--DQA1*006:01--DQB1*023:01 was found to be present only in the control population and was associated with a reduced risk of IBD ($P<0.001$). In contrast, the haplotype DLA-DRB1*015:01--DQA1*006:01--DQB1*003:01 was associated with IBD (Odds ratio [OR] =1.93, confidence interval [CI] = 1.02–3.67, $P=0.05$). This study has identified an association between DLA-type and canine IBD, supporting the immunogenetic aetiology and immunopathogenesis of this disease.

Keywords: Canine, GSD, Inflammatory bowel disease, MHC II, DLA II.

1. Introduction

Canine inflammatory bowel disease (IBD) represents a group of common chronic enteropathies characterized by persistent or recurrent gastrointestinal signs and with histological evidence of inflammation (usually lymphoplasmacytic and/or eosinophilic) in the lamina propria of the small intestine, large intestine or both (Jergens, 1999). In the United Kingdom (UK), German shepherd dogs (GSD) appear to have increased risk of developing the disease (Kathrani et al., 2011a). The exact pathogenesis of IBD in humans as well as dogs remains largely unknown, although it is believed to represent a multifactorial immune-mediated disease, resulting from a complex interplay between the intestinal adaptive and innate immune systems and the intestinal microbiome in genetically susceptible individuals (Sartor, 2006).

While the complex genetic background of human IBD has been investigated and 163 susceptibility loci identified in recent genome wide association studies (GWAS) (Jostins et al., 2012), the genetic factors associated with susceptibility to canine IBD remain largely unknown. Genetic studies in GSD have revealed Single-Nucleotide Plymoprhisms (SNPs) in genes encoding Pattern Recognition Receptors (PRR) of the innate immunity, such as TLR-4, TLR-5 and NOD-2 (Kathrani et al., 2010; Kathrani et al., 2011b; Kathrani et al., 2014). It is believed that canine IBD, similar to the situation in humans, is a complex genetic disorder with involvement of several more genetic and environmental factors that remain to be determined.

Considering the likely immunogenetic pathogenesis of canine IBD, the major histocompatibility complex (MHC) locus, also known as Dog Leukocyte Antigen (DLA) located on canine chromosome (CFA) 12 is an obvious candidate. Within the MHC class II locus, four functional genes have been identified: the monomorphic DLA-DRA1 and the highly polymorphic, DLA-DRB1, -DQA1 and DQB1. These genes encode the alpha and beta chains of MHC class II proteins that are involved in antigen presentation to CD4+ T cells. Most genetic variation in DLA class II genes occurs in exon 2, which encodes those amino acids of the peptide-binding groove of the resulting glycoproteins.

Several studies have reported associations between particular DLA class II alleles and haplotypes and susceptibility to immune mediated diseases in diseases where GSD are overrepresented, such as exocrine pancreatic insufficiency (EPI) (Tsai et al., 2013), anal furunculosis (Kennedy et al., 2008) and chronic superficial keratitis (CSK) (Jokinen et al., 2011). While significant associations have been found between human leukocyte antigen (HLA) genetic variations and IBD (Lombardi et al., 2001; Ahmad et al., 2006; Cho, 2008; Fernando et al., 2008; Lappalainen et al., 2008; Cho and Brant, 2011), this has yet to be investigated in dogs.

The aim of the current study was to therefore to investigate whether susceptibility to IBD is associated with MHC class II alleles and haplotypes in GSD.

2. Materials and methods

2.1. Ethics and welfare statement

All ethylenediaminetetraacetic acid (EDTA) blood samples used in this study were residual samples from diagnostic testing, used with informed owner consent. The use of both residual EDTA blood and buccal swab samples for this study was approved by the RVC Ethics and Welfare Committee (reference number 2013 1210).

2.2. Study population

The electronic patient records of the Queen Mother Hospital for Animals of the RVC was searched to identify GSD admitted between January 2004 and September 2014. The IBD case population was identified using stringent inclusion criteria: all cases had to have been affected by chronic (i.e. > 3 weeks duration) gastrointestinal signs. Other causes of chronic gastrointestinal disease had been excluded by a combination of routine haematology, serum biochemistry, faecal parasitology, abdominal ultrasonography, serum ACTH stimulation tests and measurement of serum canine trypsin‐like immunoreactivity concentration (TLI). Definitive diagnosis of IBD was based on histological evidence of an inflammatory infiltrate within the lamina propria of endoscopically-obtained intestinal biopsies. None of the cases suffered from concurrent diseases of known or suspected immune-mediated origin, dermatological conditions or diseases for which GSD are predisposed (i.e. anal furunculosis, atopic dermatitis, or superficial keratitis).

The same clinical database was searched to recruit the control GSD population. Dogs were only considered for inclusion as controls if they were older than 8 years of age, as IBD is typically a disease of middle age (Hall and German, 2009). None of the controls had a current or previous

history of gastrointestinal signs for a duration of more than 5 consecutive days and none of them were affected by conditions of known or suspected immune-mediated origin.

Residual EDTA blood samples were available from the RVC sample archive for a total of 56 GSD diagnosed with IBD and 44 breed-matched geriatric controls. In addition, buccal swab samples from 6 healthy GSD for which a residual blood sample was not available in the archive were provided from owners for inclusionin the control population.

Genomic DNA (gDNA) was extracted from blood using theGenElute™ Blood Genomic DNA Kit (Sigma-Aldrich®, Gillingham, UK) and from saliva samples using the Performagene® Kit (DNA Genotek Inc, Canada) according to the manufacturer's instructions.

2.3. DLA genotyping for DLA-DRB1, -DQA1 and -DQB1

The DLA specific primers obtained from previously published studies, were used to amplify exon 2 of the three polymorphic DLA class II genes. (Wagner et al., 1996a, 1996b; Kennedy et al., 2002). PCR amplification was performed in reaction volumes of 25 µl, consisting of 1 µl of gDNA, 1 µl of each specific forward and reverse primer at a concentration of 10 pmol/µl (Sigma-Aldrich), 5 µl of Hi-Spec® additive, 2.5 µl of NH_4 buffer (10× Immobuffer®), 1.15 µl of $MgCl_2$ (2.5mM final concentration) 0.25 µl of dNTPs (100 mM final concentration), 0.1 µl (1.25 IU) of Immolase® DNA polymerase (all reagents from Bioline, London, UK) and 13 µl of distilled water. PCR amplification was performed using a G-Storm® GS1 thermocycler (Gene Technologies Ltd, Essex, UK) at 95°C for 10 min, followed by 35 cycles of 94°C for 40 s, 60°C for DRB1 and DQB1 or 55°C for DQA1 for 30 s, and 72°C for 1 min, then 72°C for 10 min. PCR products were extracted using the GenElute™ PCR

Clean-Up Kit (Sigma-Aldrich) and submitted for Sanger sequencing (Source Bioscience UK Ltd, Nottingham, UK) using primer M13F. DLA alleles and haplotypes were assigned using SBTEngine® version 2.17 (GenDx, Genome Diagnostics B.V. Utrecht, Netherlands). Allele and haplotype frequencies in cases and controls were calculated and compared using VassarStats (Web Site for Statistical Computation, Vassar College, Poughkeepsie, NY, http://www.vassarstats.net/odds2x2.html). Two-way contingency tables were used to calculate two-tailed Fisher's exact probability statistic, 95 % confidence intervals, and odds ratios (OR) for association of each allele and haplotype with disease status. Statistical significance was set at a P value of < 0.05.

3. Results and Discussion

The case population included 26 male dogs and 30 female dogs (median age of 56 months; range 12-137). The median age of the control dogs was 108 months (range 96-168) and there were 23 male dogs and 27 female dogs. There was no significant difference in sex distribution between the two groups.

DLA class II alleles and haplotypes were assigned to all dogs (n=106 in total) within the IBD and control groups. 10 DLA-DRB1, 6 DLA-DQA1, and 12 DLA-DQB1 alleles were identified. Within the DLA-DRB1 alleles, DRB1*011:01 was the most common allele, with a frequency of 34%. Of the DLA-DQA1 and DLA-DQB1 alleles DQA1*006:01 and DQB1*013:02 were the most common alleles with frequencies of 40.1% and 33%, respectively (Table 1). There was a significant difference comparing cases and controls in the distribution of the alleles DRB1*015:01, DRB1*015:02 and DQB1*023:01 (Table 1).

A total of 18 different haplotypes were identified. However, only 4 of these haplotypes had a frequency greater than 5 (3 haplotypes in both groups and 1 haplotype only in the control population). The most frequent haplotypes for the whole study population were DLA-DRB1*011:01--DQA1*002:01--DQB1*013:02 (haplotype 6) present in 52.83% of dogs and DLA-DRB1*015:01--DQA1*006:01--DQB1*003:01 (haplotype 11) present in 40.6% of dogs. Table 2 shows the haplotype distribution, the number of homozygous haplotypes, and the number of dogs carrying each haplotype.

Homozygosity was more frequently seen in IBD cases (20/56, 35%) compared with controls (11/50, 22%). When the haplotype frequencies were compared between groups, a statistically significant difference was identified for 2 haplotypes (Table 2). The haplotype (No.11) DLA-DRB1*015:01--DQA1*006:01--DQB1*003:01 was more prevalent in IBD cases than in the control dogs (31.25% of the cases compared with 18.18% of the controls, OR=1.93, CI= 1.02_3.67, P=0.05) while haplotype (No.15) DLA-DRB1*015:02--DQA1*006:01--DQB1*023:01 was found in the control dogs, but was absent from the IBD case population ($P<0.001$). Although homozygosity for haplotype 11 was more common in the case population (9/56) compared with the controls (2/50), suggesting a ‘gene dosing effect’, this did not reach statistical significance (OR = 4.0, CI = 0.73–21.35, P = 0.15).

These results revealed the presence of a susceptibility haplotype (DLA-DRB1*015:01--DQA1*006:01--DQB1*003:01) and a protective haplotype (DLA-DRB1*015:02--DQA1*006:01--DQB1*023:01) for canine IBD in GSD. When the allele frequencies were compared between

IBD and control GSD (Table 1), we found that, both alleles DLA-DRB1*015:02 and DLA-DQB1*023:01 reached statistical significance suggesting a synergetic effect for the protecting haplotype.

Interestingly, the risk-associated and the protective haplotypes, differ by only two nucleotides (in codon 90 for DRB1 and in codon 13 for DQB1), causing a single amino acid change in each protein sequence. However, it is accepted that even small changes in the sequence of the peptide-binding groove of the MHC molecules could cause conformational and functional changes that impact on peptide binding and antigen presentation (Gardiner et al., 2007). An alternative explanation may be that, these particular haplotypes are in linkage disequilibrium with other disease associated gene variants nearby on CFA 12.

The potential risk haplotype, DLA-DRB1*0015:01--DQA1*006:01--DQB1*003:01 has been reported to be associated with CSK in GSD (Jokinen et al., 2011). None of the GSD in our study population had a known history or clinical signs of CSK. In contrast, the same haplotype has previously been shown to be associated with protection from anal furunculosis (Kennedy et al., 2008) in the same breed, which highlights the complex genetic nature of these disorders, as one might expect common genetic factors in IBD and anal furunculosis, both of which are immune-mediated inflammatory mucosal diseases.

It is worth noting that the risk of developing disease in genetically susceptible individuals was only moderate, as explained by the presence of the haplotype 11 in 17/50 controls (34%). Consistent with previous studies (Kennedy L.R unpublished data), haplotype 11 (DLA-DRB1*015:01--DQA1*006:01--DQB1*003:01) was the second most common haplotype for the whole study population (GSDs), second only to DLA-

DRB1*011:01--DQA1*002:01--DQB1*013:02. This moderate effect of haplotype 11 is likely due to the fact that canine IBD is a disease of complex genetic background and with environmental and epigenetic factors possibly also playing a role.

Indeed, as already mentioned, several studies suggested that the presence of specific SNPs in either surface-expressed or intracellular PRRs may also contribute to the development of IBD (Kathrani et al., 2010; Kathrani et al., 2011b; Kathrani et al., 2014). However, due to the differences in sample material, these cannot be correlated at this time point.206-210

The biological mechanism whereby DLA class II gene polymorphisms influence the immune response, potentially resulting in an immune-mediated pathology, such as in IBD is unclear. Since the polymorphisms are concentrated in exon 2, encoding the region around specific pockets of the peptide-binding groove, we speculate that they could cause conformational changes, affecting the affinity of binding of foreign peptides or causing preferential binding of peptides that either stimulate pro-inflammatory responses or diminish more regulatory mechanisms involved in mucosal tolerance. One limitation of the study was it was retrospective in nature and there was a lack of pedigree information on the dogs included in the study. It is therefore possible that some of the dogs in the IBD group were related, which would lead to an overrepresentation of certain DLA alleles and haplotypes. Future studies could also assess the association of haplotype 2 with IBD in a larger population and/or in GSDs from other geographical locations.

It has to be mentioned that we are not able to trace the breeder for the majority of the samples taken. Therefore, even it is highly unlikely given that samples were taken from dogs all over England, there is still the possibility that the majority of dogs came from the same breeder. However,

our new data, together with already identified SNPs would justify a genome wide SNP analysis of IBD affected dogs to assess whether the presence of all SNPs would impact on the onset of clinical IBD.

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Footnotes

Conflict of Interest Statement

The authors declare no conflicts of interest.

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Table 1: Frequencies of DLA class II alleles in 56 GSDs with IBD and 50 healthy controls.

DLA-	**Case**	**Control**	**Total**			
DLA-DRB1	**2n=112 (%)**	**2n=100 (%)**	**2n=212 (%)**	**OR**	**95%CI**	**P Value**
001:01	14 (12.5)	11 (11)	25 (11.79)	1.15	0.49-2.67	NS
001:02	5 (4.46)	2 (2)	7 (3.30)	2.28	0.43-12	NS
002:01	0	5 (5)	5 (2.35)	0	0-NaN	NS
006:01	1 (0.89)	0	1 (0.47)	∞	∞-NaN	NS
011:01	39 (34.82)	33 (33)	72 (33.96)	1.08	0.61-1.91	NS
012:01	6 (5.35)	6 (6)	12 (5.66)	0.88	0.27-2.84	NS
015:01	**41 (36.60)**	**23 (23)**	**64 (30.18)**	**1.93**	**1.05-3.53**	**0.03**
015:02	**3 (2.67)**	**18 (18)**	**21 (9.90)**	**0.12**	**0.03-0.44**	**0.0001**
016:01	1 (0.89)	0	1 (0.47)	∞	∞-NaN	NS
020:01	2 (1.78)	2 (2)	4 (1.88)	0.89	0.12-6.44	NS
DLA-DQA1						
001:01	19 (16.96)	13 (13)	32 (15.09)	1.36	0.63-2.93	NS
002:01	40 (35.71)	33 (33)	73 (34.43)	1.12	0.63-1.99	NS
004:01	8 (7.14)	8 (8)	16 (7.54)	0.88	0.31-2.45	NS
005:011	1 (0.89)	0	1 (0.47)	∞	∞-NaN	NS
006:01	44 (39.28)	41 (41)	85 (40.09)	0.93	0.53-1.61	NS
009:01	0	5 (5)	5 (2.35)	0	0-NaN	NS
DLA-DQB1						
001:01	0 (0)	5 (5)	5 (2.35)	0	0-NaN	NS
002:01	20 (17.85)	12 (12)	32 (15.09)	1.59	0.73-3.45	NS
003:01	38 (33.92)	20 (20)	58 (27.35)	2.05	1.09-3.84	NS
007:01	1 (89)	0	1 (0.47)	∞	∞-NaN	NS

008:02	0 (0)	1 (1)	1 (0.47)	0	0-NaN	NS
013:017	5 (4.46)	5 (5)	10 (4.71)	0.88	0.24-3.16	NS
013:02	38 (33.92)	32 (32)	70 (33.01)	1.09	0.61-1.93	NS
013:03	4 (3.57)	3 (3)	7 (3.30)	1.19	0.26-5.48	NS
017:01	0 (0)	1 (1)	1 (0.47)	0	0-NaN	NS
023:01	**5 (4.46)**	**19 (19)**	**24 (11.32)**	**0.19**	**0.07-0.55**	**0.0009**
054:01	1 (0.89)	1 (1)	2 (0.94)	0.89	0.05-14	NS
059:01	0 (0)	1 (1)	1 (0.47)	0	0-NaN	NS

In total, 10 DRB1 alleles, 6 DQA1 alleles and 12 DQB1 alleles were found in the study population. The alleles DRB1*015:01 was in a higher frequency in cases while the alleles DRB1*015:02and DQB1*023:01 were more frequent in controls. Numbers in bold indicate a significant difference between cases and controls.

Table 2: Frequencies of three loci DLA class II haplotypes in 56 cases of GSDs with IBD and 50 healthy controls.

	Haplotypes	Total	N.Haplotypes (%)					N. Homozygotes				
No.	DRB1* DQA1* DQB1*	2n=212	Case	Control	OR	CI	P value	Case	Control	OR	CI	P value
1	001:01--001:01--002:01	25 (11)	14 (12.5)	11 (11)	1.15	0.49_2.67	0.832	1	1	0.75	0.04_1.67	1
2	001:02--001:01--002:01	6 (2.8)	5 (4.46)	1 (1)	4.62	0.53_40.29	0.216	0	0			
3	001:02--001:01--008:02	1 (0.4)	0	1 (1)	0	0_Nan	0.47	0	0			
4	002:01--009:01--001:01	5 (2.3)	0	5 (5)	0	0_Nan	0.022	0	1			
5	006:01--005:011--007:01	1 (0.4)	1 (0.89)	0	∞	Nan_∞	1	0	0			
6	011:01--002:01--013:02	70 (33)	38 (33.93)	32 (32)	1.09	0.61_1.93	0.772	10	4	3.33	0.89_12.36	0.121
7	011:01--002:01--013:03	2 (0.9)	1 (0.89)	1 (1)	0.89	0.05_14.44	1	0	0			
8	012:01--004:01--013:03	1 (0.4)	1 (0.89)	0	∞	Nan_Infin	1	0	0			
9	012:01--004:01--13:017	10 (4.7)	5 (4.46)	5 (5)	0.88	0.24_3.16	1	0	0			
10	012:01--004:01--017:01	1 (0.4)	0	1 (1)	0	0_Nan	0.47	0	0			
11	**015:01--006:01--003:01[a]**	**54 (25)**	**35 (31.25)**	**19 (19)**	**1.93**	**1.02_3.67**	**0.05**	**9**	**2**	**4**	**0.73-21-35**	**0.15**
12	015:01--006:01--023:01	8 (3.7)	5 (4.46)	3 (3)	1.51	0.35_6.48	0.724	0	0			
13	015:01--006:01--054:01	2 (0.9)	1 (0.89)	1 (1)	0.89	0.05_14.44	1	0	0			
14	015:02--006:01--003:01	4 (1.8)	3 (2.68)	1 (1)	2.72	0.27_26.62	0.62	0	0			
15	**015:02--006:01--023:01[b]**	**16 (7.5)**	**0**	**16 (16)**	**0**	**0-Nan**	**<0.001**	**0**	**3**	**Nan**	**Nan-Nan**	**1**
16	015:02--006:01--059:01	1 (1.8)	0	1 (1)	0	0-Nan	0.47	0	0			
17	016:01--002:01--002:01	1 (0.4)	1 (0.89)	0	∞	Nan_∞	1	0	0			
18	020:01-- 004:01--013:03	4 (1.8)	2 (1.78)	2 (2)	0.89	0.12_6.44	1	0	0			

A total of 18 different haplotypes with frequencies >1% were identified. DLA-DRB1*015:01--DQA1*006:01--DQB1*003:01 (haplotype 11) had an increased frequency in cases and DLA-DRB1*015:02--DQA1*006:01--DQB1*023:01 (haplotype 15) was only present in controls. A P value for significance was set at 0.05 for comparison of haplotype frequencies.

Table 3: Aminoacidic sequences for DRB1 (codon 90) and DQA1 (codon13) alleles of the risk-associated and the protective haplotypes.

Haplotypes	DLA-DRB1	DLA-DQB1
Risk-associated haplotype **015:01**--006:01--**003:01**	80 90 HNYGVIESFT	10 20 DFVYQ FK**A**ECYFTNG
Protective haplotype **015:02**--006:01--**023:01**	80 90 HNYGVIESF**A**	10 20 DFVYQ FKGECYFTNG

Risk and protective haplotypes differ by only two aminoacids; T to **A** in codon 90 for DRB1 and G to **A** in codon 13 for DQB1.