Identification of susceptibility and protective major histocompatibility complex haplotypes in canine diabetes mellitus

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Abstract

Diabetes mellitus occurs spontaneously in dogs, which is believed to have an autoimmune component and to be a model of human latent autoimmune diabetes of adults (LADA). Some dog breeds (e.g. Samoyed) are particularly predisposed, whereas others (e.g. Boxer) are highly resistant. With the completion of the Dog Genome Assembly, comparative genomic studies of complex diseases in dogs, including diabetes, could provide an important investigative approach into such disorders. Type 1 diabetes in humans is strongly associated with major histocompatibility complex (MHC) class II polymorphisms. We have investigated whether canine dog leucocyte antigen (DLA) class II haplotypes are associated with diabetes. DNA from 460 cases and 1047 controls were genotyped for DLA-DRB1, DLA-DQA1 and DLA-DQB1 using sequence-based typing. Three DLA haplotypes, DRB1*009/DQA1*001/DQB1*008, DRB1*015/DQA1*0061/DQB1*023 and DRB1*002/DQA1*009/DQB1*001, were found at significantly increased frequency in cases with diabetes compared with controls. One DLA-DQ haplotype, DQA1*004/DQB1*013, was significantly reduced in cases with diabetes. Further analysis showed that DQA1 alleles carrying arginine at codon 55 of DQA1 were increased in dogs with diabetes. To our knowledge, this is the first report of a comparative study of MHC and diabetes in a non-rodent species. Since no laboratory model of LADA exists and dogs and humans share similar environments, further research into canine diabetes is warranted.

Introduction

Diabetes mellitus occurs spontaneously in domestic dogs, with an estimated prevalence of 0.32% (1). Dogs with diabetes usually present with polydipsia, polyuria and weight loss, associated with hyperglycaemia and glucosuria. Diabetes typically affects dogs aged between 5 and 12 years, and it is uncommon in juvenile animals. Oral hypoglycaemic drugs are ineffective in canine diabetes treatment, and virtually, all dogs are dependent on insulin therapy to manage their hyperglycaemia. Some breeds are predisposed to developing diabetes including the Samoyed, Tibetan terrier and Cairn terrier. In contrast, breeds such as the Boxer, German shepherd dog and Golden retriever are underrepresented in the dog population with diabetes (1, 2). These breed differences in susceptibility suggest a genetic component to the aetiology of diabetes in this species.

Canine diabetes can be classified as insulin deficiency diabetes (IDD), resulting from a congenital deficiency or acquired loss of pancreatic beta cells, or insulin resistance diabetes (IRD), resulting from hormonal antagonism of insulin function (3). There is no evidence for a canine equivalent of human type 2 diabetes, despite the fact that

obesity is common in the pet dog population. Female dogs can suffer from dioestrus diabetes during the progesterone-dominated phase of the oestrus cycle, which has a pathophysiology similar to that of human gestational diabetes (4).

Adult-onset IDD is the most common type of canine diabetes and is believed to be associated with pancreatitis and/or immune-mediated beta-cell destruction. Histopathological examination of pancreatic tissue showed insulitis to be present in around one third of dogs with diabetes, with a further third of cases showing evidence of more generalised pancreatic inflammation (5). There is evidence that autoantibodies are present in a proportion of dogs with diabetes, suggesting that autoimmunity might be involved in the pathogenesis of disease in some animals, although these could also occur as an immunological consequence of beta-cell destruction associated with pancreatitis. In one study, anti-islet cell antibodies were detected in 50% of newly diagnosed dogs with diabetes (3, 6). The antigen specificity of such anti-islet reactivity is currently unknown, although preliminary work has documented autoantibodies against canine GAD65 in 6 of 30 newly diagnosed dogs with diabetes, with two of these dogs also reacting against canine insulinoma antibody 2 (IA-2) antigen (3). The presence of autoantibodies, with the late onset and slow progression of beta-cell dysfunction in dogs with diabetes has led to speculation that canine diabetes is a model of latent autoimmune diabetes of adults (LADA). Since the dog population with diabetes contains both pedigree and crossbreed animals and pet dogs largely share the same environment as human beings, it might be possible to identify risk factors for diabetes that would not be apparent in inbred laboratory rodent species.

The domesticated dog has been vigorously line-bred over the past few hundred years to achieve an extreme phenotypic variation between different pedigrees (e.g. Great Dane *vs* Miniature poodle) but minimal genetic variation still remains between breeds. Such breeding programmes have inadvertently resulted in differences in breed susceptibility to certain endocrinopathies, including diabetes (2), hypothyroidism (7) and Addison's disease (8). With the completion of the Dog Genome Assembly and initiation of ongoing breed genetic diversity projects, comparative genomic studies in diseases, including diabetes, could provide important information into such complex genetic disorders.

In humans, the genes encoding the major histocompatibility complex (MHC) class II molecules are strongly associated with human type 1 diabetes (9) and LADA (10), accounting for over half of their genetic risk (9). Initial studies showed a strong association with HLA-DR3 and HLA-DR4 in type 1 diabetes (11). Subsequently, diabetes susceptibility was shown to be associated with the presence of an amino acid other than aspartic acid at position 57 of the HLA-DQ beta chain (DQB non-Asp57) (9) and also with the presence of arginine at position 52 of the HLA-DQ alpha chain (DQA Arg52) (12). However, the association of diabetes with MHC susceptibility alleles appears to differ in humans dependent on ethnic origin (13), which is likely to be an important consideration when attempting to identify MHC susceptibility alleles in different dog breeds.

The dog leucocyte antigen (DLA) genes are the canine equivalent of HLA. There are currently 90 DLA-DRB1, 22 DLA-DQA1 and 54 DLA-DQB1 alleles officially recognised, with extensive interbreed but minimal intrabreed DLA variation (14–16). Strong linkage disequilibrium exists between DLA class II loci with many examples of preferential allelic association. Some 'established' or conserved DLA haplotypes may be characteristic of a particular breed (15, 17). Thus, the pattern of distribution of DLA types in different dog breeds is analogous to the differences seen between different human ethnic groups and populations.

A pilot study, based on a small cohort of dogs with diabetes, suggested that at least one DLA haplotype (DRB1*009/DQA1*001/DQB1*008) was associated with an increased risk of diabetes (18). The aim of this study was to confirm and expand upon this preliminary work and identify DLA alleles/haplotypes that might confer susceptibility or resistance to diabetes in dogs.

Research design and methods

Animals

Blood samples, in ethylenediaminetetraacetic acid (EDTA) anticoagulant, from dog were submitted by veterinary practitioners to the Royal Veterinary College as part of the UK Canine Diabetes Database and Archive initiative, for measurement of haemoglobin A1c. A diagnosis of diabetes had been reached, based on consistent clinical signs with demonstration of glucosuria and persistent hyperglycaemia (blood glucose >6 mmol/l). Samples had been taken either from recently diagnosed dogs with diabetes before the commencement of therapy or from dogs undergoing treatment with insulin (Insuvet™; Schering-Plough Animal Health, Uxbridge, UK, or Caninsulin™; Intervet, Milton Keynes, UK). A total of 529 cases were collected over a period of 5 years. However, sixty-nine of the dogs with diabetes were reported to be sexually intact females at the time of diagnosis, and many of these dogs were likely to be suffering from dioestrus diabetes, which is unlikely to have an association with MHC genes. Therefore, these dogs were excluded from the analysis. Of the remaining 460 dogs with diabetes, the underlying cause of the diabetes had not been further investigated. DNA samples from control dogs without diabetes ($n = 1047$) were obtained from residual blood samples taken for diagnostic purposes at the Small Animal Hospital, University of Liverpool. All dogs with an autoimmune disease diagnosis were excluded from the control group. Table 1 shows the breakdown by breed for each of these groups of dogs. The odds ratio (OR) for risk of

Table 1 Breed numbers for the study dog groups

Breed	Insulin deficiency diabetes mellitus ($n = 460$)	Controls ($n = 1047$)
Afghan hound		2
Australian shepherd dog	1	2
Basset hound		8
Beagle	5	59
Bernese mountain dog		7
Bichon frise	11	21
Bloodhound		1
Bouvier		2
Boxer		51
Briard		3
Bull mastiff		15
Bulldog		3
Chow Chow		6
Collie (Bearded)	1	3
Collie (Border)	26	41
Collie (Rough)		5
Corgi	4	3
Dachshund (all types)	11	25
Dalmatian	1	4
Deerhound		1
Dobermann	5	36
Elkhound	1	
Foxhound		2
German shepherd dog		57
Great dane	1	6
Greyhound		2
Hovawart		6
Husky	1	12
Irish wolfhound		5
Japanese akita		4
Labrador	56	93
Lhasa apso	3	4
Lurcher	1	4
Mastiff		3
Munsterlander (Large)		2
Newfoundland	1	5
Papillon		7
Pharaoh hound		1
Pinscher (Miniature)	1	
Pointer		4
Polish lowland sheepdog	2	
Pomeranian	2	2
Poodle (All types)	8	25
Pug		1
Pyrenean mountain dog		4
Retriever (Chesapeake Bay)	1	
Retriever (Flatcoat)		2
Retriever (Golden)	6	44
Rhodesian Ridgeback	1	18
Rottweiler	4	19
Samoyed	15	9
Schnauzer (Miniature)	10	14
Setter (English)	3	33
Setter (Gordon)	3	3
Setter (Irish)	1	9
Sharpei		2
Sheepdog (Old English)	3	8
Sheepdog (Shetland)	4	3
Shih Tzu	2	19
Spaniel (CKCS)	19	17
Spaniel (Clumber)		1
Spaniel (Cocker)	15	30
Spaniel (Field)		1
Spaniel (Springer)	8	21
Spinone (Italian)		2
Spitz	1	2
St Bernard		5
Terrier (Airedale)		2
Terrier (Border)	10	11
Terrier (Boston)		1
Terrier (Bull)	1	2
Terrier (Cairn)	15	11
Terrier (Dandie Dinmont)	1	
Terrier (Fox)	1	2
Terrier (Jack Russell)	17	40
Terrier (Maltese)		2
Terrier (Manchester)		1
Terrier (Patterdale)		
Terrier (Scottish)	1	3
Terrier (Staffs Bull)	3	8
Terrier (Tibetan)	7	6
Terrier (Welsh)		1
Terrier (West Highland White)	38	33
Terrier (Yorkshire)	29	47
Vizsla Hungarian		5
Weimaraner	1	5
Whippet	1	2
X-Crossbreed	97	56

diabetes requiring insulin for each breed was calculated by comparing the canine population with diabetes with a database of UK insured dogs ($n = 47{,}000$). Breeds were assigned a risk category, dependent on the OR value (high risk = OR > 5; moderate risk = OR 2–5; neutral risk = OR 0.5–2; protected = OR < 0.5).

MHC genotyping for DLA-DRB1, DQA1 and DQB1

DNA was extracted from all samples using a standard phenol–chloroform method. DNA concentration was measured using a spectromax spectrophotometer, and samples were normalised to 20 ng/μl.

All the dogs were characterised for three DLA class II loci using either sequence-based typing (SBT) (19, 20) or reference strand-mediated conformation analysis (RSCA) (16).

All polymerase chain reactions (PCR) were performed with 25 ng DNA in a 25 μl reaction containing 1× PCR buffer, as supplied by Qiagen (with no extra magnesium),

Q solution (Qiagen, Crawley, UK), final concentrations of 0.1 μM for each primer, and 200 μM each deoxyribonucleotide triphosphate, with 2 units of Taq polymerase (Qiagen HotStarTaq). A negative control containing no DNA template was included in each run of amplifications to identify any contamination.

The primers used were DRBF forward: GAT CCC CCC GTC CCC ACA G and DRBR3 reverse: CGC CCG CTG CGC TCA, DQAin1 forward: TAA GGT TCT TTT CTC CCT CT and DQAIn2 reverse: GGA CAG ATT CAG TGA AGA GA, DQB1B forward: CTC ACT GGC CCG GCT GTC TC and DQBR2 reverse: CAC CTC GCC GCT GCA ACG TG. All primers are intronic and locus specific, and the product sizes are 303 bp for DLA-DRB1, 345 bp for DQA1 and 300 bp for DQB1.

A standard Touchdown PCR protocol was used for all amplifications, which consisted of an initial 15 min at 95°C, 14 touchdown cycles of 95°C for 30 s, followed by 1 min annealing, starting at 62°C (DRB1), 54°C (DQA1), 73°C (DQB1) and reducing by 0.5°C each cycle, and 72°C for 1 min. Then, 20 cycles of 95°C for 30 s, 55°C (DRB1), 47°C (DQA1) and 66°C (DQB1) for 1 min, 72°C for 1 min and a final extension at 72°C for 10 min.

To check for the presence of a product, 5 μl PCR product was run on a 2% agarose gel. No purification was required for RSCA. However, this was required for SBT: 2 units of shrimp alkaline phosphatase (Amersham, Little Chalfont, UK) and 10 units of Exo1 (New England Biolabs, Hitchin, UK) were added to 5 μl of PCR product. The mixture was incubated for 1 h at 37°C, then for 15 min at 80°C.

RSCA: fluorescent-labelled references (FLRs) were generated from a range of DLA-DRB1 alleles from the domestic dog and grey wolf. The FLRs were produced by PCR using cloned alleles as templates and a 5′-FAM22-labelled forward primer. To increase the proportion of the labelled reference strand in the reaction, the primer proportions were altered to 0.5 μM FAM22-labelled forward primer and 0.1 μM reverse unlabelled primer. All other aspects of the PCR reaction remained the same. This method of generating the FLR increases the heights of the FLR allele heteroduplex peaks relative to the homoduplex peaks in RSCA (data not presented). All the resulting FLRs were diluted 1:30 in water before use in the hybridisation reactions.

To form duplexes between test samples and FLRs, 2 μl of diluted FLR and 2 μl of test sample PCR product were mixed in a 96-well plate and incubated in a thermal cycler at 95°C for 10 min, ramped down to 55°C at 1°C/s, 55°C for 15 min and 4°C for 15 min. The plate was stored at 4°C until required. Subsequently, 8 μl of distilled water was added to each hybridisation reaction, and 2 μl was mixed with 7.8 μl water and 0.2 μl Genescan Rox-500 size standards (Applied Biosystems, Warrington, UK) in a 384-well plate. These samples were run on an ABI 3100 DNA analyser, using 50-cm capillary arrays, 4% Genescan non-denaturing polymer (Applied Biosystems), and data were collected using matrix Dye set D. The conditions were injection voltage 15kV, injection time 15 s, run voltage 15kV and run temperature 30°C. Each run took 35 min. The data were analysed using 'GENESCAN' and 'GENOTYPER' software (Applied Biosystems). GENESCAN was used to assign sizes to each peak, based on the ROX-500 standards. Using GENOTYPER, the allele peaks formed by the control samples were assigned to 'bins' for each FLR used. The bins were exported into an in-house program, which assigned the alleles for each sample.

Haplotype assignment and statistical methods

Three-locus, DLA-DRB1/DQA1/DQB1, haplotypes were identified by following a sequential analytical process. First, all dogs that were homozygous at all three loci were selected, and from these several different DLA-DRB1/DQA1/DQB1 haplotype combinations were identified. Dogs that were homozygous at only two loci were then selected. From these dogs, many of the previous haplotypes were confirmed and also several additional haplotypes were identified. The remaining dogs were examined using the haplotype data already identified, and haplotypes were assigned to each of these dogs. From these dogs further possible haplotypes were identified. This method of assigning haplotypes is possible because of the high frequency of dogs homozygous for all three DLA class II loci (approximately 35%) and the limited haplotype distributions within each breed. Maximum likelihood methods were not used as they would give less accurate results.

Further analyses were based on methods adapted from Svejgaard et al. (21), as used by the International Histocompatibility workshops (22).

Results

DLA alleles and haplotypes were assigned to all of the dogs with diabetes and control dogs. During the study, one new DLA-DRB1 allele, DRB1*00203, (accession number AM075472) was identified in several Dachshunds.

A range of DLA-DRB1/DQA1/DQB1 haplotypes was observed in the dog population studied. For each of these haplotypes, the percentage of animals carrying at least one copy was compared between dogs with diabetes and control dogs. Three DLA haplotypes DLA-DRB1*009/DQA1*001/DQB1*008, DRB1*015/DQA1*006/DQB1*023 and DRB1*002/DQA1*009/DQB1*001 were found to be significantly increased in frequency in dogs with diabetes compared with control dogs without diabetes (Table 2). In contrast, one DLA-DQ haplotype, DQA1*004/DQB1*013, was significantly reduced in dogs with diabetes compared with control dogs (16.5% *vs* 23.1%, OR = 0.66, 95% confidence interval (CI) = 0.49–0.88, $P < 0.005$). The dataset was divided into males and females, and these

Table 2 DLA haplotype frequencies in dogs with diabetes and control dogs without diabetes

DRB1	DQA1	DQB1	Diabetic dogs ($n = 460$)	Frequency %	Controls ($n = 1047$)	Frequency %	Odds ratio	95% confidence interval	*P* value
001	001	002	96	20.9	219	20.9			NS
001	001	036	10	2.2	20	1.9			NS
001	003	004	4	0.9	17	1.6			NS
001	009	001	10	2.2	12	1.2			NS
002	009	001	54	11.7	85	8.1	1.51	1.03–2.19	0.03
004	002	015	1	0.2	44	4.2			NS
005	003	005	5	1.1	13	1.2			NS
006	004	013	7	1.5	39	3.7			NS
006	005	007	81	17.6	180	17.2			NS
006	005	02001	8	1.7	24	2.3			NS
008	003	004	10	2.2	21	2.0			NS
009	001	008	55	12.00	65	6.2	2.05	1.38–3.04	0.0002
011	002	013	29	6.3	66	6.3			NS
012	001	002	2	0.4	14	1.3			NS
012	004	013	8	1.7	24	2.3			NS
012	004	013017	37	8.0	105	10.0			NS
013	001	002	40	8.7	70	6.7			NS
015	006	003	7	1.5	27	2.6			NS
015	006	019022	11	2.4	8	0.8			NS
015	006	02002	25	5.4	37	3.5			NS
015	006	022	13	2.8	14	1.3			NS
015	006	023	158	34.4	268	25.6	1.52	1.19–1.94	0.0006
015	009	001	13	2.8	52	5.0			NS
018	001	002	12	2.6	21	2.0			NS
018	001	008	4	0.9	32	3.1			NS
020	004	013	25	5.4	77	7.4			NS
023	003	005	12	2.6	13	1.2			NS
Rare haplotypes			48	10.4	149	14.2			NS

NS, not significant.

were analysed separately to assess the affect of gender. One haplotype, DLA-DRB1*002/DQA1*009/DQB1*001, was increased in males compared with females (38/242, 15.7% in males and 16/211, 7.6% in females), but all other haplotype frequencies were unaffected.

Given the suggested role of HLA-DQ amino acid sequence variability in human type 1 diabetes, further analysis was performed, showing that DLA-DQA1 alleles, specifically coding for an arginine residue at position 55 (Arg55) of the DQ alpha chain, were increased in frequency in dogs with diabetes compared with controls (84.5% *vs* 74.8%, OR = 1.82, 95% CI = 1.35–2.45, $P < 0.00005$). However, there was no obvious association with the presence or absence of an aspartic acid residue at position 57 of the DLA-DQ beta chain.

Since there is considerable interbreed variability in DLA haplotype frequencies, further analysis was performed by stratifying breeds according to their diabetes risk status, in an attempt to determine whether haplotypes shared by different breeds would segregate with the different risk groups. In the highest risk breed, the Samoyed, the DRB1*009/DQA1*001 haplotype was not only associated with the DQB1*008 allele but also with DQB1*002 and DQB1*046. Furthermore, the DRB*015/DQA1*006/DQB*019/DQB1*022 haplotype, which carries two DQB1 alleles, has only been found in the Samoyed breed to date. To allow comparisons to be made between high-risk breeds and since no clear association with susceptibility had been found with DQB alleles, it was decided to focus on DRB1*009/DQA1*001 and DRB1*015/DQA1*006 two-locus haplotypes. In the high-risk group (Samoyed, Cairn terrier and Tibetan terrier), similar genotypes were seen across the pedigrees (Table 3). In contrast, the DRB1*009/DQA1*001 haplotype was not present in the protected group (Golden Retriever, German Shepherd Dog and Boxer), although the DRB1*015/DQA1*006 homozygous genotype was present in 16 of 152 (10.5%) control dogs in the protected group.

The frequency of dogs carrying the DRB1*009/DQA1*001 susceptibility haplotype and DQA1*004/DQB1*13 protective haplotype was examined for cases and controls in each risk group (Table 4). Although when stratified in this way, the sample size for most individual breeds was too small for robust analysis, two observations could be made. First, the frequency of the DRB1*009/DQA1*001 haplotype was generally higher in those breeds assigned to the higher risk categories. Second, the reverse

Table 3 Genotype frequencies in dogs with diabetes from high-risk breeds

Haplotype 1		Haplotype 2		Samoyed ($n = 15$)	Cairn terrier ($n = 15$)	Tibetan terrier ($n = 7$)	Total high risk ($n = 37$)
DRB1*	DQA1*	DRB1*	DQA1*				
009	001	009	001	1	2	0	3
015	006	015	006	4	8	3	15
009	001	015	006	5	2	1	8
009	001	x	x	2	0	0	2
015	006	x	x	3	3	2	8
x	x	x	x	0	0	1	1

x, any other haplotype.

was generally observed for the DQA1*004/DQB1*13 protective haplotype (Figure 1A).

When the percentage of dogs carrying DQA1 alleles containing Arg55 was examined, 100% of animals (for both cases and controls) in the highest risk group were positive. The frequency of Arg55-positive DQA1 alleles gradually reduced as the diabetes risk reduced with a higher number of affected dogs being positive than controls (Figure 1B).

Discussion

Although a diagnosis of diabetes in dogs is easy to establish, with the exception of middle-aged female dogs that develop diabetes during dioestrus, most dogs with diabetes could be classified as type 1B (idiopathic). In this study, it was not possible to subdivide the dog population with diabetes into autoantibody-positive and autoantibody-negative groups since the human serological assays do not discriminate between dog with diabetes and control dogs and the canine GAD65 and canine IA-2 radio-immunoassays have only recently been developed (3). Therefore, it was decided to perform DLA genetic analysis in the dog population with diabetes as a whole, even though this is likely to contain dogs with diabetes that is not immune mediated.

Despite analysing a heterogeneous population of dogs with diabetes, this study found that the risk of disease is

Table 4 Percentage of dogs from selected breeds with a high risk and a protective haplotype

					DRB1*009/DQA1*001 haplotypes				DQA1*004/DQB1*013 haplotypes			
			Diabetic	Control	Diabetic		Control		Diabetic		Control	
Risk group	Breed risk	Breed	*n*	*n*	*n*	%	*n*	%	*n*	%	*n*	%
High	17.30	Samoyed	15	9	8	53.3	5	55.6	4	26.7	2	22.2
High	6.93	Tibetan terrier	7	6	1	14.3	2	33.3	0	0	0	0
High	6.77	Cairn terrier	15	11	5	33.3	3	27.3	0	0	0	0
Moderate	3.60	Bichon frise	11	21	0	0	3	14.3	0	0	0	0
Moderate	3.48	Yorkshire terrier	29	47	0	0	0	0	0	0	2	4.3
Moderate	3.18	Miniature Schnauzer	10	14	7	70.0	13	92.8	0	0	0	0
Moderate	2.89	Border collie	26	41	3	11.5	3	7.3	2	7.7	3	7.3
Moderate	2.83	Dachshund	11	25	7	63.6	4	16.0	0	0	2	8
Moderate	2.51	Border terrier	10	11	0	0	0	0	0	0	1	9.1
Moderate	2.40	Miniature poodle	8	25	1	12.5	1	4.0	0	0	0	0
Neutral	1.74	Rottweiler	4	19	0	0	0	0	0	0	0	0
Neutral	1.70	WHW terrier	38	33	0	0	0	0	0	0	0	0
Neutral	1.48	Jack Russell terrier	17	40	3	17.7	7	17.5	0	0	3	7.5
Neutral	1.45	CKC Spaniel	19	17	8	42.1	7	41.2	8	42.1	2	11.8
Neutral	1.22	Dobermann	5	36	0	0	1	2.8	5	100	36	100
Neutral	0.97	Labrador	56	93	0	0	3	3.2	25	44.6	58	62.4
Neutral	0.78	Crossbreed	97	56	13	13.4	5	8.9	18	18.6	11	19.6
Neutral	0.75	Cocker spaniel	15	30	0	0	0	0	0	0	1	3.3
Protected	0.19	Golden retriever	6	44	0	0	0	0	5	83.3	37	84.1
Protected	0.15	German shepherd dog	0	57	0	0	0	0	0	0	8	14
Protected	0.07	Boxer	0	51	0	0	0	0	0	0	3	5.9

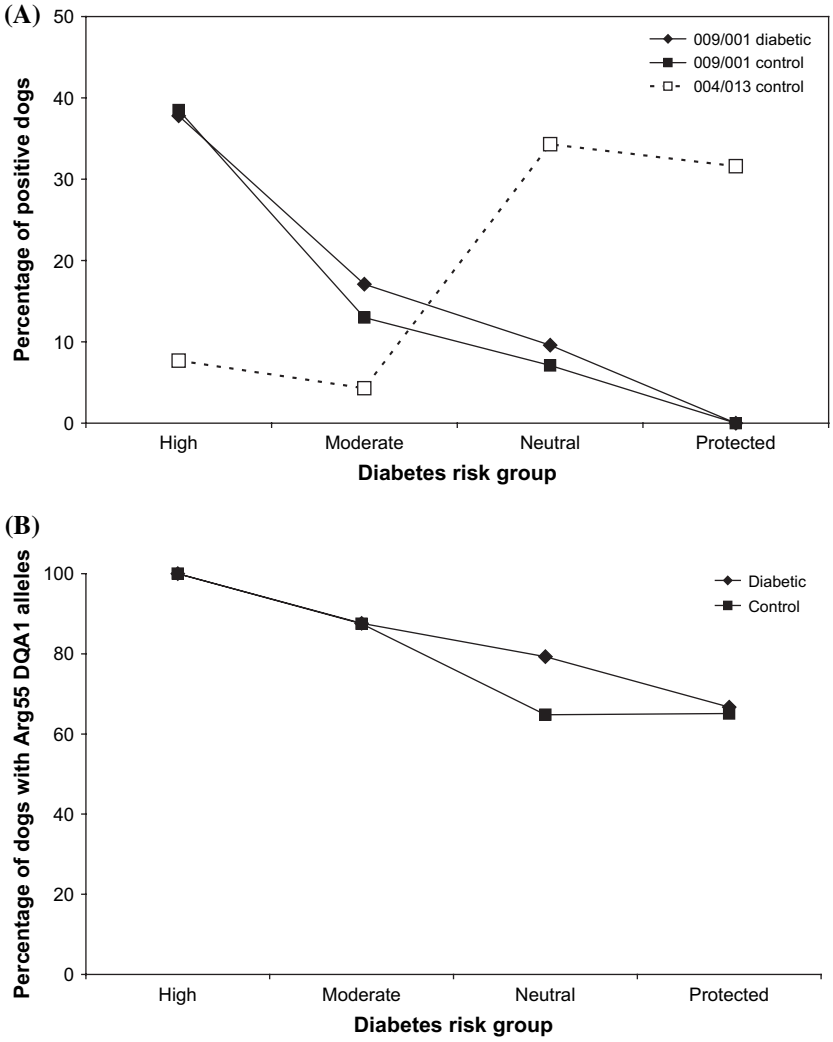

Figure 1 Frequency of dogs carrying susceptibility and protective haplotypes (A) and the reduction in Arg55 DQA1 alleles with decreased risk group (B). In B, the diabetic-resistant breeds are not shown since $n < 10$.

associated with DLA class II haplotypes: DRB1*009/DQA1*001/DQB1*008, DRB1*015/DQA1*006/DQB1*023 and DRB1*002/DQA1*009/DQB1*001. In contrast, the DLA-DQA1*004/DQB1*013 haplotype appears to confer protection against diabetes. These haplotypes may actually identify susceptible or resistant breeds, rather than individuals. These data provide evidence that there is a genetic component to the aetiology of canine diabetes that could explain the breed differences in susceptibility to the disease. Together with preliminary evidence that autoantibodies are present in a proportion of dogs with diabetes (3), this also supports the proposal that diabetes can be immune mediated in this species.

The genetic make-up of the pet dog population presents a unique challenge for investigation of the genetics of canine disease. The population with diabetes and control population in this study contained outbred animals and pedigree dogs representing a wide variety of different breeds. Since there is considerable interbreed but often minimal intrabreed variability, in DLA haplotypes, this can influence genetic analysis. Carrying out a case–control study was problematic in terms of low numbers in some breeds, particularly those in the highest risk group, which are not particularly common in the UK. However, since diabetes is likely to be a complex genetic disorder with additional environmental risk factors, even with larger numbers, there may not be significant differences in DLA haplotypes comparing cases and controls of the same breed, particularly where there has been rigorous inbreeding. Using the NOD mouse as an example, there is no genetic difference between animals that develop diabetes and those that do not; yet, there is clearly an MHC association with the disease. It would be interesting to study cases and control littermates of pedigree animals. However, such research requires the cooperation of dog breeders and the acknowledgement that a potential genetic risk factor exists in the bloodline.

The breed profile of the diabetic dog population shows marked differences in diabetes risk from the Samoyed (with an OR of 17.3) to the Boxer (with an OR of 0.07). This range of diabetes risk across breeds is reminiscent of what is seen in different human populations where disease prevalence can be extremely high in some ethnic groups originating from a limited gene pool (23). In particular, diabetes and other autoimmune conditions are very prevalent in a number of discrete human populations such as indigenous North Americans, where there are exceptionally raised frequencies of high-risk HLA alleles and haplotypes (24, 25). To minimise breed-specific bias, we chose to analyse the data in groups of breeds stratified into diabetes risk groups ranging from high risk (e.g. Samoyeds, Tibetan terriers, and Cain terriers) through to breeds exhibiting clear protection (e.g. Boxers, German shepherd dogs and Golden Retrievers). Interestingly, when the DRB1*009/DQA1*001 haplotype is examined across such risk groups, the frequency is greatest in the high-risk group, where it is present in all three breeds, and lowest in the protected group, where this haplotype is absent. The association with this haplotype is unlikely to have occurred as the result of breed bias in the population as the three high-risk breeds are unrelated and the high-risk group as a whole is still outnumbered by Labradors with diabetes, a popular breed where this haplotype is absent and which are neutral in terms of disease risk.

Polymorphism within MHC genes ensures diverse immune response in a population. However, selective inbreeding of dogs can lead to restriction of DLA haplotypes in some pedigrees, which clearly will have impact on immune responses and consequently with susceptibility to immune-mediated disease. The Samoyed, which is by far the most diabetes-susceptible breed, has an interesting DLA profile, with haplotypes that are only found in this breed. Both DRB1*009/DQA1*001 and DRB1*015/DQA1*006 haplotypes are present in this breed with 5 of 15 Samoyed dogs with diabetes being heterozygous for these high-risk haplotypes, reminiscent of the association with HLA-DR3/-DR4 heterozygosity in type 1 diabetes. There are four Samoyed dog with diabetes that express the protective DQA1*004/DQB1*013 haplotype, although these are heterozygous with a high-risk haplotype, suggesting that the DRB1*009/DQA1*001 and DRB1*015/DQA1*006 susceptibility haplotypes are dominant, in contrast to the situation in human type 1 diabetes where HLA-DR2 is dominantly protective. We propose that most Samoyed dogs are susceptible to diabetes by virtue of the high-risk DLA haplotypes common within this breed, but that disease will only occur if other genetic and environmental factors come into play.

It is also interesting to note that diabetes is uncommon in German shepherd dogs and rare in the Boxer breed. German shepherd dogs are more susceptible to other conditions, including deep pyoderma, small intestinal bacterial overgrowth and systemic aspergillosis, whereas Boxer dogs are susceptible to a range of different cancers, suggestive of an underlying immune defect in these two breeds. The restricted DLA haplotypes present in German shepherd dogs and Boxers could be the reason why such breeds are more susceptible to infection or neoplasia, but this could result in them being less susceptible to autoimmune beta-cell destruction. The obvious divergence in diabetes susceptibility between Boxers and Samoyeds also suggests that extended cross-bred pedigrees could provide an important inroad into identifying quantitative trait loci through linkage studies.

The molecular basis for the HLA association with type 1 diabetes is linked to variability in amino acids at key positions in the MHC class II protein. Amino acid differences at position 57 of the DQ beta chain and position 52 of the DQ alpha chain can have impact on presentation of antigenic peptides, which in turn will influence immune responses and tolerance. Although there did not appear to be any association with the presence or absence of aspartic acid at position 57 on the canine DQ beta chain, there was an association with DQA alleles that coded for arginine at position 55 (DQA1*00101, *00601, *00901, *01001, *01301, *01401 and *01501). This positively charged amino acid is likely to have impact on peptide binding to the canine MHC molecule and is comparable to the HLA-DQ alpha Arg52 association in human diabetes. Recent studies have also shown reproducible associations of the DLA-DQA1*00101 allele (Arg55 positive) with susceptibility to canine hypothyroidism in Dobermanns and other breeds (26, 27). As this allele is also associated with canine diabetes, the possibility exists that this constitutes a common risk factor for autoimmunity in the dog. It is also interesting to note that DLA-DRB1*00901 carries the 'shared epitope'. This is a sequence of five amino acids in the third hypervariable region of DRB1 exon 2, which has been associated with rheumatoid arthritis not only in the humans (28) but also in the dog (29).

We investigated the possibility that dogs with diabetes could present with multiple endocrinopathies, associated with autoimmunity. None of the 460 dogs with diabetes had concurrent hypothyroidism (based on low T4 with high endogenous thyroid stimulating hormone measurements), although this has been reported elsewhere (30). To identify any dogs with latent autoimmune thyroid disease, we measured canine thyroglobulin autoantibody levels in more than 200 diabetic serum samples, but none was positive. In contrast, there were five dogs with diabetes with concurrent Addison's disease (based on a deficiency of cortisol following adrenocorticotrophic hormone stimulation). These rare cases might be equivalent to human autoimmune polyendocrine syndrome (APS) type 2. Four of the five dogs expressed an Arg55-positive DQA allele (the fifth was

a Rottweiler, a breed that has a very restricted DLA profile, with only two haplotypes identified to date). Additionally, two of the five dogs expressed the DRB1*009/DQA1*001/DQB1*008 high-risk haplotype.

To the authors' knowledge, this is the first report of a comparative study into MHC genetics and diabetes in a non-rodent species. As in human diabetes, it is likely that MHC genes only contribute a proportion of the total genetic susceptibility to the disease, and further work is required to identify other susceptibility genes. There is currently no laboratory animal model of LADA or APS type 2 and since pet dogs share an environment similar to that of human beings, the study of canine diabetes provides an opportunity to investigate the interaction between genetic and environmental factors involved in the pathogenesis of diabetes that is not possible in laboratory animal models.

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