Original article

Breed differences in development of anti-insulin antibodies in diabetic dog and investigation of the role of dog leukocyte antigen (DLA) genes.

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Abstract

Administration of insulin for treatment of diabetes mellitus in dogs can stimulate an immune response, with a proportion of animals developing anti-insulin antibodies (AIA). For an IgG antibody response to occur, this would require B cell presentation of insulin peptides by major histocompatibility complex (MHC) class II molecules, encoded by dog leukocyte antigen (DLA) genes, in order to receive T-cell help for class switching. DLA genes are highly polymorphic in the dog population and vary from breed to breed. The aim of the present study was to evaluate AIA reactivity in diabetic dogs of different breeds and to investigate whether DLA genes influence AIA status.

Indirect ELISA was used to determine serological reactivity to insulin in diabetic dogs, treated with either porcine or bovine insulin preparations. DLA haplotypes for diabetic dogs were determined by sequence-based typing of *DLA-DRB1, -DQA1* and *-DQB1* loci. Significantly greater insulin reactivity was seen in treated diabetic dogs (n = 942) compared with non-diabetic dogs (n = 100). Of the diabetic dogs treated with a bovine insulin preparation, 52.3% (182/348) were AIA positive, compared with 12.6% (75/594) of dogs treated with a porcine insulin preparation, suggesting that bovine insulin is more immunogenic. Breeds such as dachshund, Cairn terrier, miniature schnauzer and Tibetan terrier were more likely to develop AIA, whereas cocker spaniels were less likely to develop AIA, compared with crossbreed dogs. In diabetic dogs, DLA haplotype *DRB1*0015--DQA1*006--DQB1*023* was associated with being AIA positive, whereas the haplotype *DLA-DRB1*006--DQA1*005--DQB1*007* showed an association with being AIA negative. These research findings suggest that DLA genes influence AIA responses in treated diabetic dogs.

Keywords: Diabetes mellitus; Dog breeds; Susceptibility genes; Dog leukocyte antigen; Major histocompatibility complex; Insulin

Introduction

Diabetes mellitus is one of the most common endocrine disorders in dogs, with an estimated prevalence of 0.32% in the UK (Davison et al., 2005). It is a disease of middle to late age, with the majority of dogs diagnosed between 7 and 12 years old. It has been proposed that there are several potential underlying causes of diabetes in dogs, including immune mediated destruction of the beta cells of the pancreas, chronic pancreatitis and insulin resistance due to hormonal antagonism (Hoenig, 2002; Rand et al., 2004). Certain breeds of dog are predisposed to developing diabetes, which strongly suggests that there is a genetic component to disease susceptibility (Catchpole et al., 2008). Breeds such as the Samoyed, Tibetan terrier and Cairn terrier have an increased risk of developing diabetes, whereas other breeds, such as the Boxer and German Shepherd Dog have a reduced risk (Catchpole et al., 2005).

Dog leukocyte antigen (DLA) genes, which encode MHC class II molecules, demonstrate considerable inter-breed variability (Kennedy et al., 2002) and have been linked with susceptibility to diabetes mellitus (Catchpole et al., 2008; Kennedy et al., 2006). Three DLA haplotypes in particular, *DLA-DRB1*009--DQA1*001--DQB1*008, DRB1*015--DQA1*006--DQB1*023* and *DRB1*002--DQA1*009--DQB1*001*, have been shown to be associated with susceptibility to diabetes and are prevalent in the Samoyed, Tibetan terrier and Cairn terrier breeds.

Virtually all diabetic dogs require insulin by injection to control their hyperglycaemia, but this can stimulate an immune response and some dogs develop anti-insulin antibodies (AIA) following initiation of therapy (Davison et al., 2003). A previous study showed that around

60% of dogs treated with Insuvet lente (Intervet / Pfizer Animal Health), a bovine insulin preparation, developed AIA, whereas only around 10% of dogs treated with Caninsulin (MSD Animal Health), a porcine insulin preparation, developed AIA (Davison et al., 2008). This suggests that bovine insulin is more immunogenic than porcine insulin, which is likely due to variation of the insulin sequence between species (Fineberg et al., 2007); the amino acid sequence is the same comparing porcine and canine insulin, but bovine insulin differs from canine insulin by two amino acids in the A chain (Davison et al., 2003). Although bovine insulin seems to be more immunogenic in dogs, the AIA that develop are not species specific as they react with both porcine and bovine insulin in ELISA and typically recognise conformational, rather than linear epitopes (Davison et al., 2003).

Generation of an immunoglobulin (Ig)G antibody response to a foreign protein requires B cells to process antigen and present digested peptide fragments, bound to MHC class II molecules, to recruit T cell help. Polymorphisms in MHC class II genes influence the structure of the peptide-binding groove and therefore the repertoire of antigenic peptides that can be presented to the immune system. Considering the small size of the insulin molecule, it is likely that there are limited peptide epitopes available for presentation, suggesting that MHC class II genes could play a major role in whether an anti-insulin response is initiated or not (Fineberg et al., 2007). In humans, differences in AIA production have been associated with particular human leukocyte antigen (HLA) types (Reeves et al., 1984; Schernthaner et al., 1979; Sklenar et al., 1982) and in mice the presence of specific H-2 linked immune response genes for insulin has been demonstrated (Kapp and Strayer, 1978).

The aim of the present study was to further evaluate AIA in diabetic dogs, treated with different insulin preparations, to determine whether there were breed differences in AIA reactivity and

to evaluate whether there was any evidence for a DLA genetic influence on AIA responses in diabetic dogs.

Materials and methods

Blood samples

Blood samples (serum and EDTA blood) from diabetic dogs were collected between 2002 and 2010 as part of the UK Canine Diabetes Register at the Royal Veterinary College. This archive was established with institutional ethical approval, by recruiting blood samples from diabetic dogs seen by first opinion veterinary practices and referral centres throughout the UK, with informed owner consent. The population used in this study consisted of 109 recently diagnosed and untreated diabetic dogs, 594 diabetic dogs treated with Caninsulin (MSD Animal Health) and 348 dogs treated with Insuvet lente (Intervet and latterly Pfizer Animal Health). Diabetic dogs in the insulin-treated groups had been receiving insulin therapy for more than 30 days. One hundred control serum samples were obtained from non-diabetic dogs referred to the Queen Mother Hospital for Animals at the Royal Veterinary College, following completion of diagnostic testing, with informed owner consent for residual samples to be used in clinical research.

ELISA procedure

Measurement of AIA in diabetic and control serum samples was performed by indirect ELISA, as described previously (Davison et al., 2008). Briefly, flat bottomed 96 well microtitre plates (Maxisorp, Nunc) were coated with porcine insulin (Sigma-Aldrich) at 10 µg/mL in 0.05 M carbonate/ bicarbonate buffer, pH 9.6 (Sigma-Aldrich) overnight at 4 °C. All washing steps were performed with phosphate buffered saline (Invitrogen) supplemented with 0.1% tween 20

(Sigma-Aldrich) (PBST). Plates were blocked with PBST supplemented with 2% skimmed milk (Marvel, Premier beverages) and 10% rabbit serum (Sigma-Aldrich). Serum was added in duplicate wells, diluted 1:100 in PBST supplemented with 1% skimmed milk and 10% rabbit serum. Antibody binding was detected using a rabbit anti-canine IgG HRP conjugate (Stratech Scientific) diluted 1:10,000 in PBST supplemented with 1% milk and 10% rabbit serum. Plates were developed using 3,3′,5,5′-tetramethylbenzidine substrate (TMB slow kinetic form, Sigma-Aldrich), which was incubated for 1–2 min before reactions were stopped using 2 M sulphuric acid (Scientific Laboratory Supplies). The optical density was measured at 450 nm (OD_{450nm}) using a SpectraMAX M2 plate reader (Molecular Devices).

ELISA data analysis

Results for each serum sample were calculated as the mean OD_{450nm} for anti-insulin reactivity minus the background (obtained from wells coated with 50 µL 0.05 M carbonate/bicarbonate buffer without insulin). A positive control serum sample identified in a pilot study was used in all subsequent ELISA to allow for correction of inter-assay variability. This was used to calculate a correction factor, which was applied to normalise all OD_{450nm} values on each plate. A threshold for positive reactivity was set using non-diabetic control samples. A minimum threshold of mean + 1.96× SD was used.

Sequence-based typing

Diabetic dogs were characterised for three MHC class II loci *DLA-DRB1, -DQA1* and *–DQB1*, using sequence-based typing. Genomic DNA was extracted from EDTA blood samples using the QIAamp DNA Blood Midi Kit (Qiagen), according to the manufacturer's instructions. Polymerase chain reaction (PCR) was performed with 25 ng genomic DNA in a 25 µL reaction containing 1× PCR buffer, Q solution, 200 µM each dNTP, 2.5 units HotStarTaq DNA

polymerase (all from Qiagen) and primers at a final concentration of 0.1 µM each. The primers used were DLA-DRBIn1forward: 5′-CCG TCC CCA CAG CAC ATT TC-3′, DLA-DRBIn2-T7 reverse: 5′-TAA TAC GAC TCA CTA TAG GG TGT GTC ACA CAC CTC AGC ACC A-3′, DLA-DQAIn1forward: 5′-TAA GGT TCT TTT CTC CCT CT-3′, DLA-DQAIn2 reverse: 5′-GGA CAG ATT CAG TGA AGA GA-3′, DLA-DQB1B-T7 forward: 5′-TAA TAC GAC TCA CTA TAG GG CTC ACT GGC CCG GCT GTC TC-3′ and DLA-DQBR2 reverse: 5′-CAC CTC GCC GCT GCA ACG TG-3′. All primers were intronic and locus specific, and produced amplicons of 303 bp (DRB1), 345 bp (DQA1) and 300 bp (DQB1). A standard touchdown PCR protocol was employed for all amplifications. This consisted of an initial activation at 95°C for 15 min, then 14 touchdown cycles of 95°C for 30 s (denaturation), a 1 min annealing step starting at 62°C (DRB1), 54°C (DQA1), 73°C (DQB1) and reducing by 0.5°C each cycle, and an elongation step at 72°C for 1 min. This was followed by 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.

Prior to sequencing, the presence of a product was checked by agarose gel electrophoresis, and PCR products were purified by adding 2 units of shrimp alkaline phosphatase (Amersham Biosciences) and 10 units of Exonuclease1 (New England Biolabs) to 5 µL of PCR product. The mixture was incubated for 1 h at 37°C and then for 15 min at 80°C. Sequencing reactions, using a T7 primer for DLA-DRB1 and DQB1, and DQAIn2 reverse primer for DLA-DQA1, were performed using Big Dye Terminator V3 (Life Technologies). Samples were sequenced on an Applied Biosystems 373 Genetic Analyser and sequencing data was analysed using SBTengine (GenDX).

Statistical analysis

Statistical analyses were performed using commercial software package (SPSS version 18 for Windows, IBM). Anti-insulin antibody reactivity in control and diabetic groups was compared using Kruskal-Wallis test, which if significant was followed by multiple Mann-Whitney *U* tests with a Bonferroni correction applied. Anti-insulin antibody status in different breeds of dog was examined using Fisher's exact test to compare the ratio of AIA negative and AIA positive dogs in each pedigree breed with the cross breed population. Odds ratios were calculated to compare DLA haplotype frequencies between AIA negative and positive diabetic dogs, Fisher's exact test was then used to determine whether the differences were significant.

Results

Anti-insulin antibodies (AIA) were measured by ELISA in serum samples from 100 control dogs, 109 newly diagnosed diabetic dogs and 942 treated diabetic dogs. Of the treated diabetic dogs, 594 had received treatment with Caninsulin and 348 others had received Insuvet lente. The threshold for positive AIA reactivity was to be set using the 95% confidence interval of the control population (mean $OD_{450nm} \pm 1.96\times$ SD); however, since the control dogs were all found to have negligible anti-insulin reactivity (mean OD_{450nm} = 0.001; SD = 0.005), an arbitrary ELISA absorbance value of 0.1 was used as the threshold for AIA positivity (Figure 1A). There was a significant difference in AIA reactivity comparing newly diagnosed diabetic dogs and control dogs ($P = 0.02$), with three of the 109 newly diagnosed diabetic dogs classified as positive for AIA (Figure 1A). Anti-insulin reactivity in the treated diabetic dogs was significantly greater than in control and newly diagnosed diabetic dogs ($P < 0.001$). Diabetic dogs treated with bovine insulin demonstrated significantly greater AIA reactivity than dogs treated with porcine insulin ($P < 0.001$). In dogs treated with porcine insulin, 12% (75/594) were AIA positive, compared with 52% (182/348) of dogs treated with bovine insulin (Fig.

1B). Although the cases and controls were not entirely aged matched, age was not found to be an influencing factor on the development of AIA, since treated diabetic dogs that were AIA positive were of a similar age (mean = 9.9 years; range 0.4–17.6 years) to those that were AIA negative (mean = 10.1 years; range 1.0–16.3 years).

Upon submission of samples to the UK Canine Diabetes Register veterinary practitioners were asked to provide information for each dog relating to insulin dose, frequency and duration of treatment. Fructosamine concentrations were measured in serum samples to provide information on glycaemic control. For each of these variables, comparisons were made between AIA negative and AIA positive dogs for the type of insulin treatment used. Since, information was lacking for some dogs, a smaller population was taken forward for this analysis (Table 1).

The majority of diabetic dogs (731/914) were receiving twice daily injections of insulin, with the remainder receiving once daily injections. Dogs receiving once daily insulin were receiving greater insulin doses per injection than those treated twice daily (Table 1). There was also a trend for dogs who had developed AIA to be receiving greater insulin doses than those who were AIA negative, but this was only significant in dogs treated with the bovine insulin product ($P = 0.017$). The duration of insulin treatment varied greatly within the diabetic population, but there appeared to be no relationship between duration of insulin treatment and the AIA status (Table 1). Fructosamine values were similar, comparing the different treatment groups, with no significant difference between groups (Table 1).

Within the population of diabetic dogs treated with either Caninsulin or Insuvet lente, AIA status was assessed, comparing different dog breeds. Breed differences were seen in terms of the antibody response to insulin treatment (Figures 2A, B). Breeds such as miniature schnauzer

and Tibetan terrier were found to be relatively susceptible to developing AIA, when treated with either insulin preparation. The dachshund and Cairn terrier were found to be more likely to develop AIA when treated with Insuvet lente, when compared with crossbreed dogs. Cocker spaniels were relatively resistant to developing AIA when treated with insulin, compared with other breeds. The Samoyed breed was unusual, in that treatment with Caninsulin was found to stimulate AIA in a greater proportion of dogs, compared with crossbreed dogs, but the proportion of AIA positive dogs was similar to that seen in other breeds when Insuvet lente was used.

Genomic DNA was extracted from EDTA blood samples from diabetic dogs and DLA genotyping was performed. Not all the dogs that had been assayed for AIA had an EDTA sample available, so a smaller population of dogs was used for this analysis. After DLA genotyping the diabetic dog population, the data were grouped according to insulin type and AIA status (Table 2; Table 3). DRB1*0015:01--DQA1*006:01--DQB1*023:01 was found to be associated with being AIA positive in dogs treated with porcine insulin, whereas DRB1*006:01--DQA1*005:011--DQB1*007:01 was found to be associated with being AIA negative in both porcine and bovine insulin treated diabetic dogs. The intention was to evaluate the relationship between AIA status and DLA-type in several breeds, but only Labrador retrievers had sufficient numbers of dogs of defined haplotypes to be able to perform this analysis in a robust way (Table 4; Table 5). DLA-DRB1*001:01--DQA1*001:01--DQB1*002:01 was found to be associated with being AIA negative in Labrador retrievers treated with bovine insulin.

Discussion

This study was designed to investigate anti-insulin antibodies in diabetic dogs, and to examine the influence that breed and DLA genes might have on the antibody responses elicited by insulin therapy. Porcine insulin was selected as the antigen for use in ELISA as it had been shown previously that there was a high correlation between AIA reactivity measured against bovine or porcine insulin and that there was antibody cross-reactivity between these two insulin types (Davison et al., 2003)

Control dogs demonstrated negligible AIA reactivity, which might be expected since they have not been exposed to the antigen, over and above that present physiologically. However, a previous study reported AIA in 4 of 120 control dogs (Davison et al., 2008). This latter finding might represent false positives in the insulin ELISA, or might be due to the presence of insulin autoantibodies in dogs that were potentially in a pre-diabetic state. Insulin autoantibodies are associated with the development of diabetes in NOD mice (Abiru et al., 2001) and in humans their presence has been shown to be predictive for diabetes when used in conjunction with other islet-cell antibodies (Franke et al., 2005). In the present study, 3 of 109 newly diagnosed diabetic dogs were positive for AIA, suggesting that insulin autoantibodies might be present as a component of the disease process, but that these are relatively uncommon.

Anti-insulin antibody reactivity in diabetic dogs treated with Insuvet lente was significantly greater than that seen in Caninsulin-treated dogs, where 52% of Insuvet lente-treated dogs were AIA positive, compared with 12% of dogs receiving Caninsulin. These findings are consistent with a previous study in a much smaller sample population (Davison et al., 2008). This difference in anti-insulin antibody response is likely due to the fact that bovine insulin (Insuvet lente) differs from canine insulin by two amino acids and would be seen by the host immune

system as foreign antigen, whereas porcine insulin (Caninsulin) has an identical amino acid sequence compared with canine insulin, so should be less immunogenic. However, if this was the only factor affecting the AIA response, it might be expected that all dogs treated with Insuvet lente would be AIA positive and all dogs treated with Caninsulin would be AIA negative. Since this is not the case, host immune factors must also play a role in determining the immunological outcome (tolerance or activation) when dogs receive insulin therapy.

The development of an antibody response to self or harmless environmental antigen is usually prevented by several tolerance mechanisms. Although B cell tolerance is not particularly robust, class switching to IgG and production of high antibody titres are dependent upon the presence of CD4+ T-helper cells. Central tolerance to self-antigens is established by clonal deletion of T cells during their development in the thymus. In humans, insulin expression in the thymus is influenced by a variable number of tandem repeats (VNTR) in the insulin gene promoter (Pugliese et al., 1997; Vafiadis et al., 1997). Long VNTR alleles (140-210 repeats), have been shown to confer resistance to diabetes which is associated with higher levels of insulin mRNA expression in the thymus compared to short VNTR alleles (26-63 repeats), which predispose to diabetes. This suggests that the higher levels of insulin expression associated with the long *INS* VNTR alleles allow induction of immune tolerance and so protects against the development of diabetes caused by auto reactive T cells. In dogs, no equivalent VNTR has been found in the canine insulin gene promoter, although one has been identified in intron 2 of the gene (Catchpole et al., 2013). However, this location in the insulin gene means that it is unlikely to have the same effect on expression of canine insulin and induction of immunological tolerance.

Peripheral tolerance mechanisms become important, once T cells have left the thymus and enter the circulation, migrating through the various secondary lymphoid tissues. Clonal ignorance can occur when antigen is presented at a level insufficient to stimulate an immune response. Since expression of insulin in the periphery is restricted to the beta cells of the pancreas and insulin in the circulation has a relatively short half-life (Duckworth et al., 1998), it is possible that the immune system's exposure to insulin as an antigen fails to reach the threshold for lymphocyte activation. Other forms of peripheral tolerance include T cell anergy, when antigen is presented without the presence of co-stimulatory molecules, and active suppression, mediated by regulatory T cells.

In dogs treated with porcine insulin that develop AIA, it is possible that there has been a failure to establish tolerance to insulin, and immune activation occurs when an exogenous insulin preparation is administered. In contrast, Insuvet lente-treated dogs who fail to develop AIA are presumably already tolerant to insulin (by central and/or peripheral tolerance mechanisms) or establish tolerance after repeated injection of exogenous foreign insulin. A previous study has shown that dogs treated with an escalating dose of mixed bovine-porcine insulin failed to produce an anti-insulin antibody response (Menzel et al., 1971), which might be related to induction of anergy in insulin-specific naïve T cells or stimulation of regulatory T cells.

The duration of insulin treatment did not appear to have an effect on the presence or absence of AIA (Table 1). A previous study found that dogs varied in their AIA response over time (Davison et al., 2008). In some cases, AIA were produced within 3 months of initiating insulin therapy, reaching a plateau at around 6 months. However, there were some individuals who showed no evidence of insulin reactivity during the first year of treatment. A similar pattern has also been observed in human diabetic patients (Reeves and Kelly, 1982).

The majority of diabetic dogs were receiving twice daily insulin injections and were generally on a lower dose per kg per injection than those receiving once daily insulin therapy (Table 1). Twice daily therapy has been shown to provide better glycaemic control than once daily insulin administration (Hess and Ward, 2000). Dogs receiving once daily injections of bovine insulin, that were AIA positive, were more likely to be receiving higher doses of insulin, compared with those that were AIA negative. It is possible that exposure to greater amounts of insulin per injection influences the magnitude of the antibody response. Alternatively, it is feasible that the AIA are having a partial neutralising effect on insulin activity, which would therefore require higher doses of insulin to maintain glycaemic control. In human diabetic patients, it has been shown that high levels of circulating AIA can affect the dose of insulin required to maintain glycaemic control and that by changing to a less immunogenic insulin there is a reduction in AIA and the dose of insulin required by the patient (Walford et al., 1982).

Measurement of serum fructosamine is routinely used to evaluate glycaemic control in diabetic dogs (Webb, 2002). Fructosamine values were similar and not significantly different comparing the different insulin treatment groups and comparing dogs of different AIA status. This suggests that the presence of AIA does not substantially influence glycaemic control per se, although this might be one factor to consider in an individual unstable diabetic dog, where insulin resistance is suspected.

Different breeds of dog were found to vary in their immune response to insulin therapy. Breeds such as the miniature schnauzer and Tibetan terrier were found to be relatively susceptible to developing AIA, whereas Cocker spaniels were found to be less likely to develop AIA when treated with insulin. Samoyed dogs were relatively susceptible to developing AIA when treated with Caninsulin, but were no more likely to do so, compared with other breeds, when treated

with Insuvet lente. This was unexpected since this suggests that self-insulin is as immunogenic as foreign insulin in this particular breed. Samoyeds are one of the most diabetes-susceptible dog breeds in the UK (Catchpole et al., 2005) and this research finding might be consistent with an autoimmune pathogenesis.

These differences in AIA reactivity, comparing dog breeds, suggest that genetic factors might influence the immune response to insulin. Different strains of mice have been shown to vary in their immune response to insulin and this is believed to be controlled by the H-2 linked immune response (Ir) genes of the MHC (Kapp and Strayer, 1978; Keck, 1975). In mice immunised with porcine or bovine insulin, those with the *H-2*^{b} haplotype respond only to porcine insulin, whereas those with the *H-2*^{d} haplotype respond to both types. *H-2*^{b} mice present epitopes from the insulin A-chain, which differs between porcine and bovine insulin by two amino acids, whereas *H-2*^{d} mice present insulin B-chain epitopes, which are identical in porcine and bovine insulin (Keck, 1975; Rosenwasser et al., 1979).

The current study examined the possibility that polymorphisms in *DLA* genes might influence antigen presentation and therefore immune responses to insulin in dogs. Two *DLA* haplotypes showed an association with AIA status. *DRB1*0015:01--DQA1*006:01--DQB1*023:01* was associated with being AIA positive in Caninsulin-treated dogs, whereas *DRB1*006:01--DQA1*005:011--DQB1*007:01* showed an association with being AIA negative regardless of the insulin type used, suggesting that some DLA-types do influence AIA responses in treated diabetic dogs. The haplotype that was associated with development of AIA has also been shown to be associated with overall susceptibility to diabetes in dogs (Kennedy et al., 2006), suggesting it might play a role in presentation of pancreatic auto-antigens (including insulin) involved in the pathogenesis of the disease. The haplotype which is associated with being

negative for AIA is one which is commonly found in Cocker spaniels. This suggests that either this MHC haplotype is unable to present insulin epitopes or, alternatively, that it can present insulin peptides, but that this leads to efficient tolerance mechanisms preventing an antibody response from being stimulated. Peptide binding studies using purified canine MHC molecules, similar those undertaken for *HLA-DR* (O'Sullivan et al., 1990) and *HLA-DQ* (Kwok et al., 1995), could potentially be used to investigate this further.

The associations between DLA and AIA status that were seen in the diabetic population as a whole were not evident in Labrador retrievers, although when stratified according to AIA status and DLA-type, the numbers of dogs in each category were relatively low. The DLA haplotype *DRB1*001:01--DQA1*001:01--DQB1*00201* showed an association with being AIA negative in Insuvet lente-treated diabetic Labradors, suggesting that some associations between DLA and AIA reactivity might be breed specific or that other genes are also involved in determining whether AIA develop or not.

Conclusion

The current study has demonstrated that treatment of diabetic dogs with Insuvet lente was more likely to stimulate an AIA response compared with treatment using Caninsulin. Both breed of dog and DLA haplotype were found to influence the development of AIA, suggesting that genetic factors are involved in determining whether a dog will make an immune response to insulin during therapy.

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Table 1. Data relating to insulin therapy and glycaemic control in AIA negative and positive dogs.

	Insulin dose SID treatment (IU/kg/injection)	Insulin dose BID treatment (IU/kg/injection)	Duration of treatment (months)	Fructosamine (μmol/L)
Porcine insulinAIA negative	1.08[0.29–4.07]n = 80	0.70[0.04–2.61]n = 408	8.60[1–78.4]n = 515	477.5[189–992]n = 464
Porcine insulinAIA positive	1.33[0.46–3.27]n = 13	0.71[0.33–1.56]n = 58	9.55[1.2–81.4]n = 74	491.5[216–940]n = 70
Bovine insulinAIA negative	1.05*[0.43–4.6]n = 40	0.82[0.29–2.72]n = 106	10.60[1–104.2]n = 162	501[204–903]n = 131
Bovine insulinAIA positive	1.40*[0.55–3.17]n = 39	0.87[0.12–2.78]n = 127	7.55[1.3–59.0]n = 182	512[247–940]n = 145

Data is shown as the median, [range] and number of cases (n). SID = once daily. BID = twice daily. Data significantly associated with AIA status is shown in bold (*$P < 0.05$).

Table 2. DLA haplotypes in diabetic dogs treated with Caninsulin in relation to AIA status

	AIA negative		AIA positive			
DLA haplotype [a]	Number	Frequency (%)	Number	Frequency (%)	OR	*P* value
001:01--001:01--002:01	115	14.97	12	10.34	0.690	NS
001:01--001:01--036:01	18	2.34	1	0.86	0.367	NS
001:01--003:01--004:01	5	0.65	2	1.72	2.646	NS
001:01--009:01--001:01	24	3.12	2	1.72	0.551	NS
002:01--009:01--001:01	52	6.77	4	3.44	0.506	NS
006:01--004:01--013:03	5	0.65	0	0	0	NS
006:01--005:011--007:01	**97**	**12.63**	**7**	**6.03**	**0.477**	**0.043**
006:01--005:011--020:01	13	1.69	1	0.86	0.509	NS
008:02--003:01--004:01	7	0.91	0	0	0	NS
009:01--001:01--008:02	56	7.29	9	7.75	1.063	NS
011:01--002:01--013:02	16	2.08	0	0	0	NS
011:01--002:01--013:03	9	1.17	1	0.86	0.735	NS
012:01--004:01--013:03	8	1.04	2	1.72	1.653	NS
012:01--004:01--013:017	32	4.16	11	9.48	2.279	NS
013:01--001:01--002:01	26	3.38	2	1.72	0.509	NS
015:01--006:01--003:01	11	1.43	2	1.72	1.203	NS
015:01--006:01--011:01	7	0.91	0	0	0	NS
015:01--006:01--019:054	9	1.17	6	5.17	4.419	NS
015:01--006:01--020:02	30	3.91	4	3.44	0.880	NS
015:01--006:01--022:01	8	1.04	0	0	0	NS
015:01--006:01--023:01	**68**	**8.85**	**22**	**18.97**	**2.144**	**0.0015**
015:01--009:01--001:01	16	2.08	0	0	0	NS
015:02--006:01--023:01	45	5.85	13	11.21	1.916	NS
018:01--001:01--002:01	8	1.04	1	0.86	0.827	NS
018:01--001:01--008:02	13	1.69	3	2.58	1.527	NS
020:01--004:01--013:03	29	3.77	3	2.58	0.684	NS
023:01--003:01--005:01	7	0.91	0	0	0	NS
040:01--010:01--019:01	5	0.65	0	0	0	NS
Other haplotypes	29	3.78	8	6.98		
Total number	768		116			

[a] DLA haplotype for DRB1*--DQA1*--DQB1*. OR, odds ratio; NS, not significant. *P* values were calculated using Fisher's exact test. DLA haplotypes significantly associated with AIA status are shown in bold.

Table 3. DLA haplotypes in diabetic dogs treated with Insuvet lente in relation to AIA status.

	AIA negative		AIA positive			
DLA haplotype [a]	Number	Frequency (%)	Number	Frequency (%)	OR	*P* value
001:01--001:01--002:01	27	9.44	37	11.14	1.180	NS
001:01--001:01--036:01	1	0.35	7	2.11	6.030	NS
001:01--003:01--004:01	1	0.35	7	2.11	6.030	NS
001:01--009:01--001:01	3	1.05	4	1.20	1.143	NS
002:01--009:01--001:01	19	6.64	19	5.72	0.861	NS
006:01--004:01--013:03	4	1.40	9	2.71	1.936	NS
006:01--005:011--007:01	**43**	**15.03**	**22**	**6.63**	**0.441**	**0.0009**
006:01--005:011--020:01	2	0.70	4	1.20	1.714	NS
008:02--003:01--004:01	2	0.70	3	0.90	1.286	NS
009:01--001:01--008:02	14	4.90	30	9.04	1.845	NS
011:01--002:01--013:02	4	1.40	3	0.90	0.643	NS
011:01--002:01--013:03	7	2.45	4	1.20	0.490	NS
012:01--004:01--013:03	4	1.40	4	1.20	0.857	NS
012:01--004:01--013:017	16	5.59	15	4.52	0.809	NS
013:01--001:01--002:01	16	5.59	11	3.31	0.592	NS
015:01--006:01--003:01	1	0.35	6	1.81	5.171	NS
015:01--006:01--011:01	1	0.35	4	1.20	3.429	NS
015:01--006:01--019:054	11	3.85	6	1.81	0.470	NS
015:01--006:01--020:02	9	3.15	6	1.81	0.575	NS
015:01--006:01--022:01	3	1.05	8	2.41	2.295	NS
015:01--006:01--023:01	34	11.89	47	14.16	1.191	NS
015:01--009:01--001:01	1	0.35	5	1.51	4.314	NS
015:02--006:01--023:01	13	4.55	28	8.43	1.853	NS
018:01--001:01--002:01	8	2.80	2	0.60	0.214	NS
018:01--001:01--008:02	6	2.10	1	0.30	0.143	NS
020:01--004:01--013:03	10	3.50	9	2.71	0.774	NS
023:01--003:01--005:01	5	1.75	3	0.90	0.514	NS
040:01--010:01--019:01	2	0.70	4	1.20	1.714	NS
Other haplotypes	19	6.64	24	7.23		
Total number	286		332			

[a] DLA haplotype for DRB1*--DQA1*--DQB1*. OR, odds ratio; NS, not significant. *P* values were calculated using Fisher's exact test. DLA haplotypes significantly associated with AIA status are shown in bold.

Table 4. DLA haplotypes in diabetic Labrador retrievers treated with Caninsulin in relation to AIA status.

	AIA negative		AIA positive			
DLA haplotype [a]	Number	Frequency (%)	Number	Frequency (%)	OR	*P* value
001:01--001:01--002:01	17	25.76	1	5.26	0.204	NS
002:01--009:01--001:01	1	1.52	0	0	0	NS
006:01--005:011--007:01	5	7.57	2	10.53	1.391	NS
006:01--005:011--020:01	5	7.57	0	0	0	NS
008:02--003:01--004:01	1	1.52	0	0	0	NS
009:01--001:01--008:02	0	0	1	5.26	0	NS
012:01--004:01--013:017	18	27.27	7	36.84	1.351	NS
012:01--004:01--013:03	1	1.52	1	5.26	3.460	NS
015:01--006:01--023:01	11	16.67	2	10.53	0.632	NS
015:02--006:01--023:01	6	9.09	4	21.05	2.316	NS
020:01--004:01--013:03	1	1.52	1	5.26	3.460	NS
Total number	66		19			

[a] DLA haplotype for DRB1*--DQA1*--DQB1*. OR, odds ratio; NS, not significant. *P* values were calculated using Fisher's exact test. DLA haplotypes significantly associated with AIA status are shown in bold.

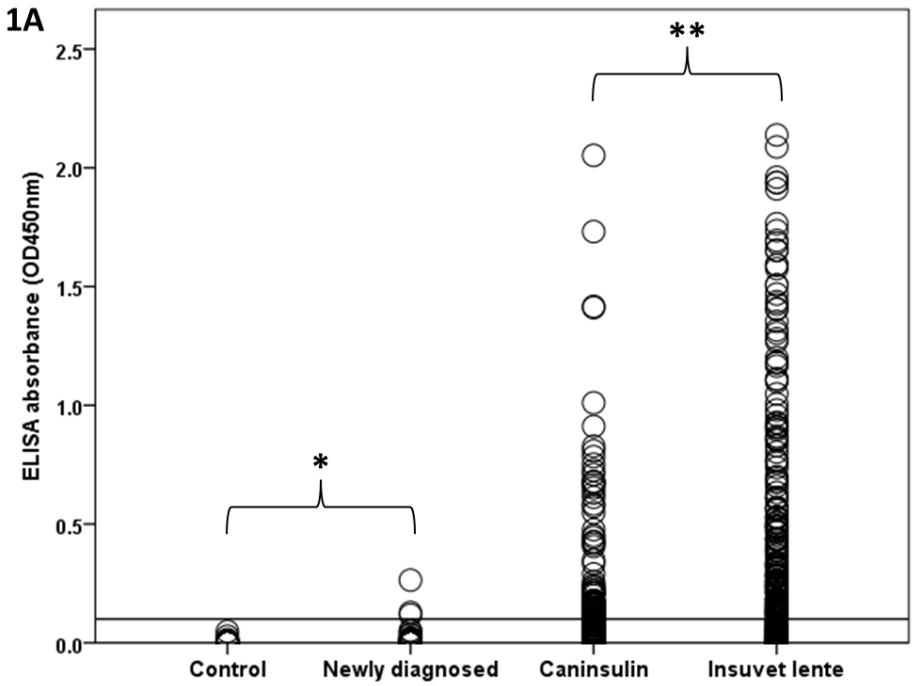
Table 5. DLA haplotypes in diabetic Labrador retrievers treated with Insuvet lente in relation to AIA status.

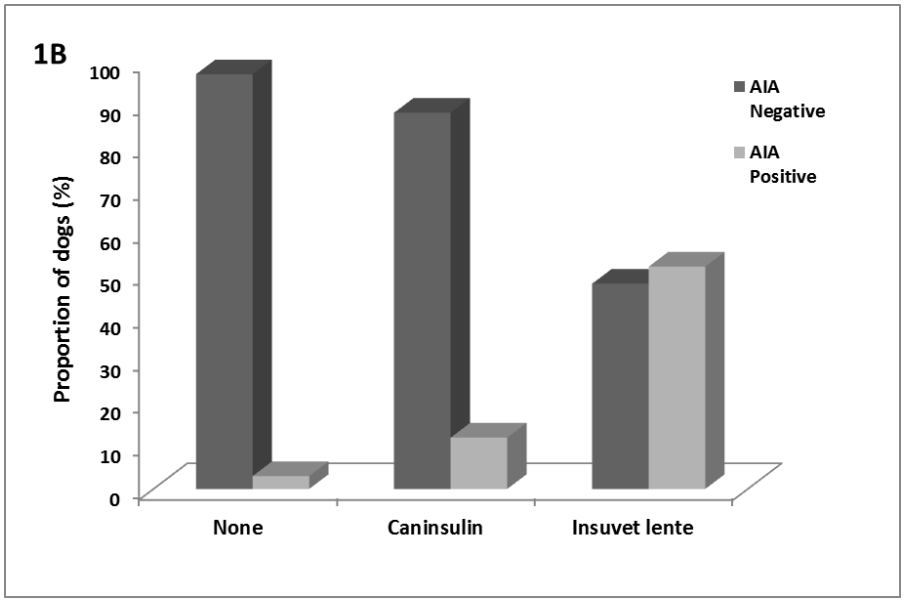
	AIA negative		AIA positive			
DLA haplotype [a]	Number	Frequency (%)	Number	Frequency (%)	OR	*P* value
001:01--001:01--002:01	**10**	**23.81**	**1**	**2.63**	**0.110**	**0.008**
001:01--001:01--036:01	0	0	1	2.63	0	NS
006:01--005:011--007:01	9	21.43	5	13.16	0.614	NS
006:01--005:011--020:01	1	2.38	1	2.63	0	NS
008:02--003:01--004:01	1	2.38	2	5.26	2.210	NS
011:01--002:01--013:01	0	0	1	2.63	0	NS
012:01--004:01--013:017	10	23.81	11	28.95	1.216	NS
012:01--004:01--013:03	0	0	2	5.26	0	NS
012:01--001:01--002:01	0	0	1	2.63	0	NS
013:01--001:01--002:01	0	0	1	2.63	0	NS
015:01--006:01--023:01	3	7.14	4	10.53	1.475	NS
015:02--006:01--023:01	6	14.29	6	15.79	1.105	NS
019:01--004:01--013:03	1	2.38	0	0	0	NS
020:01--004:01--013:03	1	2.38	2	5.26	2.210	NS
Total number	42		38			

[a] DLA haplotype for DRB1*--DQA1*--DQB1*. OR, odds ratio; NS, not significant. *P* values were calculated using Fisher's exact test. DLA haplotypes significantly associated with AIA status are shown in bold.

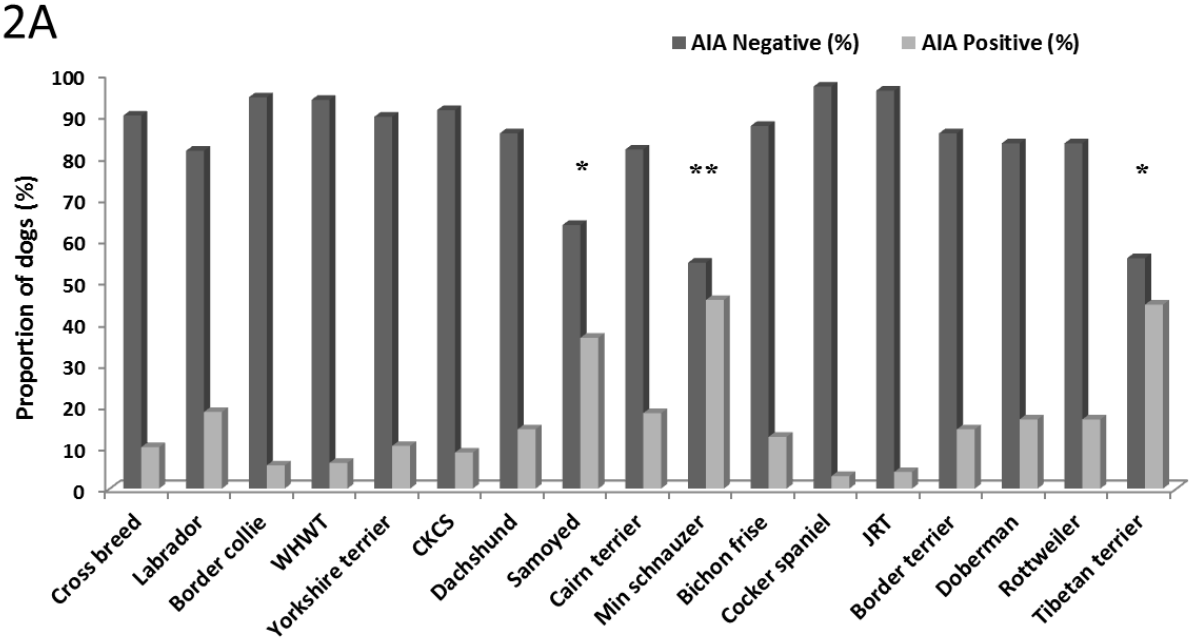
Fig. 1. Anti-insulin antibodies in diabetic and control dogs. (A) Anti-insulin antibodies (AIA) were measured by ELISA in serum samples from 100 control dogs, 109 newly diagnosed diabetic dogs and 992 diabetic dogs treated with either Caninsulin or Insuvet lente. Each data point represents the normalised ELISA absorbance value for each serum sample and the line represents the threshold for AIA positivity (OD_{450nm} = 0.1). *P* values were calculated using Mann-Whitney *U* tests with a Bonferroni correction. **P* <0.05; ***P* <0.001. (B) Bar chart showing the proportion of diabetic dogs positive or negative for AIA in the different treatment groups.

Fig. 2. Anti-insulin antibody reactivity in different dog breeds. Diabetic dogs treated with (A) Caninsulin or (B) Insuvet lente were grouped according to breed and AIA status. *P* values were generated using Fishers exact test to compare each pedigree breed with the reference cross-breed population. *****P* <0.05, ***P* <0.01.

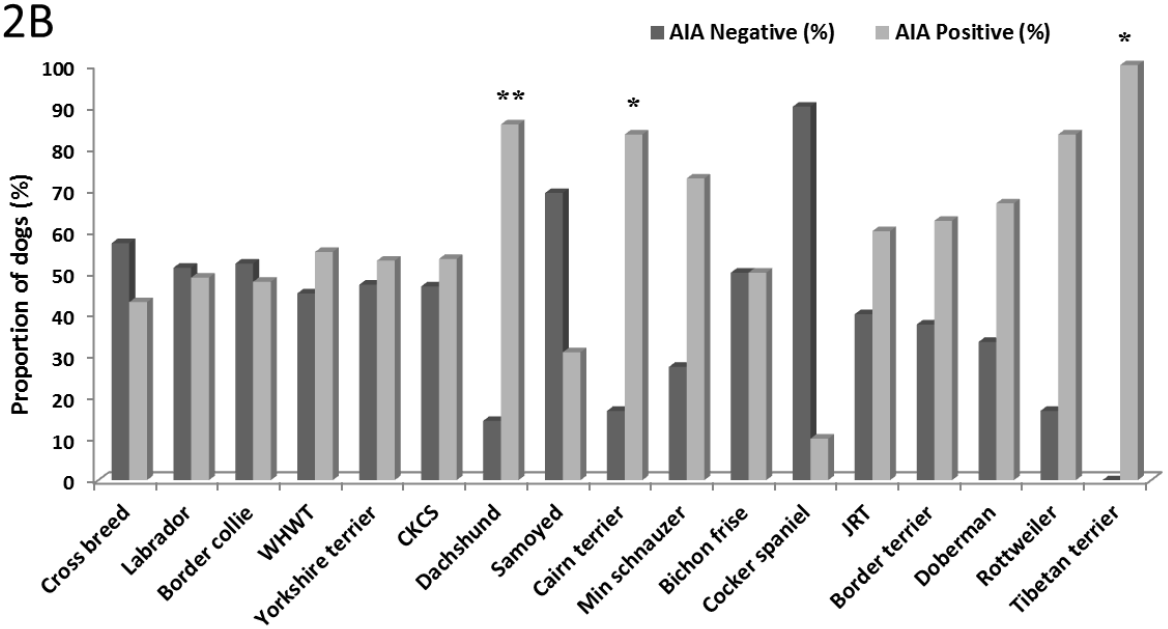

1A
ELISA absorbance (OD450nm)
2.5
2.0
1.5
1.0
0.5
0.0
*
**
Control
Newly diagnosed
Caninsulin
Insuvet lente


1B
Proportion of dogs (%)
100
90
80
70
60
50
40
30
20
10
0
AIA Negative
AIA Positive
None
Caninsulin
Insuvet lente

2A


AIA Negative (%)
AIA Positive (%)
Proportion of dogs (%)
100
90
80
70
60
50
40
30
20
10
0
*
**
*
Cross breed
Labrador
Border collie
WHWT
Yorkshire terrier
CKCS
Dachshund
Samoyed
Cairn terrier
Min schnauzer
Bichon frise
Cocker spaniel
JRT
Border terrier
Doberman
Rottweiler
Tibetan terrier

2B


AIA Negative (%)
AIA Positive (%)
Proportion of dogs (%)
100
90
80
70
60
50
40
30
20
10
0
**
*
*
Cross breed
Labrador
Border collie
WHWT
Yorkshire terrier
CKCS
Dachshund
Samoyed
Cairn terrier
Min schnauzer
Bichon frise
Cocker spaniel
JRT
Border terrier
Doberman
Rottweiler
Tibetan terrier