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Inconsistent MHC Class II association in Beagles experimentally infected with Leishmania infantum

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Abstract

The clinical outcome of *Leishmania infantum* infection in dogs varies from subclinical infection to severe disease. Researchers attribute this variability in clinical manifestations to the ability of the immune response to limit pathogen multiplication and dissemination, which is, in part, likely determined by the immune response genes. The aim of this study was to test the hypothesis that MHC class II genes are associated with disease outcome of experimental *L. infantum* infection in Beagles. Dog leukocyte antigen (DLA) class II haplotypes were characterised by sequence-based typing of Beagle dogs experimentally infected with *L. infantum* during vaccine challenge studies. Variability of response to infection was determined by clinical score, serology and quantification of *L. infantum* DNA in the bone marrow over the study period.

Dogs showed limited DLA diversity and the DLA profiles of dogs recruited for the different vaccine challenge studies differed. There were variable responses to infection, despite the apparent restriction in genetic diversity. One haplotype DLA-DRB1*001:02--DQA1*001:01--DQB1*002:01 was associated with increased anti-*Leishmania* antibodies in one infection model, but no DLA associations were found in other groups or with parasite load or clinical score. Examination of this particular DLA haplotype in a larger number of dogs is required to confirm whether an association exists with the immune or clinical responses to *L. infantum* infection.

Keywords: Beagle; DLA; Genetics; Leishmaniosis; MHC
Introduction

The protozoan parasite *Leishmania infantum* is most commonly transmitted between mammalian hosts via biting female sandflies, belonging to the genera *Phlebotomus* or *Lutzomyia*. The distribution of such vectors largely limits infection to particular geographical regions (Killick-Kendrick, 1999). *Leishmania infantum* is endemic in the Mediterranean basin, Central and South America and parts of Asia and Africa (Palatnik-de-Sousa and Day, 2011). Europe has approximately 2.5 million infected dogs, based on seroprevalence data, (Moreno and Alvar, 2002) although no official surveillance system for recording the number of dogs infected exists.

Canine leishmaniosis presents with diverse clinicopathological abnormalities and clinical outcomes. Furthermore, there appears to be significant individual variation following infection with the *L. infantum* parasite with only some dogs developing clinical disease (Baneth et al., 2008). In an experimental infection model utilised in vaccine studies, high doses of amastigotes or promastigotes are given IV, but even under these circumstances, some dogs do not develop clinical signs over the study period, despite a relatively large challenge dose (Campino et al., 2000; Costa et al., 2013).

The non-specific clinical signs and the absence of a reference standard test complicate the diagnosis of canine leishmaniosis (Rodriguez-Cortes et al., 2010). Serological testing is often used for diagnostic purposes, to monitor the infection course and/or the response to treatment. However, while *Leishmania*-specific antibody levels do not correlate with disease protection, high antibody reactivity is associated with clinical disease (Reis et al., 2006). Detection of *Leishmania* DNA in the tissues with PCR is a sensitive alternative technique for
identifying infection (Cortes et al., 2004) and high parasite load in the tissues is associated with clinical disease (Dos-Santos et al., 2008).

Previous canine studies appeared to confirm a role for T-cell mediated immunity (CMI) in resistance to canine leishmaniosis, with IFN-γ, produced by stimulated lymphocytes from subclinically infected dogs, able to lyse Leishmania infected macrophages, in contrast with lymphocytes from clinically infected dogs (Pinelli et al., 1995). Despite several studies examining cell mediated immunity in dogs, a clear picture of the T-helper phenotypes associated with disease outcome has not emerged and results are often contradictory (Hosein et al., 2017). Therefore, CMI assays are infrequently performed to diagnose clinical leishmaniosis and their utility for predicting outcome of infection is not always reliable.

The genetic background of the host might play a role in determining the outcome of infection with L. infantum and differences in susceptibility between different dog breeds has been suggested. The Ibizan hound in particular has been identified as a potentially resistant breed (Solano-Gallego et al., 2000). Other studies have suggested that the Cocker spaniel and Boxer breeds might be more at risk of developing clinical disease (Franca-Silva et al., 2003). As the outcome to L. infantum infection is largely dependent on the host immune response, much of the genetic research has focussed on immune response genes that might determine the outcome of infection.

Dog leukocyte antigen (DLA) class II genes determine antigen presentation by MHC class II molecules and influence the subsequent immune response; therefore, they might also determine the ability to control L. infantum parasite numbers in tissues and clinical outcome. A previous study has examined DLA genes in a naturally infected group of cross breed dogs
in Brazil and DLA-DRB1 015:02 was associated with increased risk of *L. infantum* infection (Quinnell et al., 2003). The impact of the DLA background of laboratory Beagle dogs on the response to experimental infection, undertaken as part of vaccine efficacy studies, has not been examined and immunogenetic profiling of dogs enrolled in vaccine challenge studies might provide valuable additional information in terms of the response to vaccination and the clinical outcome following experimental infection. In this study, DLA class II genes were examined in four groups of Beagle dogs experimentally challenged with *L. infantum* and studied post-challenge as part of vaccine studies.

**Materials and methods**

**Study population**

EDTA blood samples were taken as part of ongoing commercial studies into candidate vaccines. Blood sampling was undertaken by appropriately trained Zoetis staff in accordance with the relevant regulatory approval at the partner institution. Approval for use of residual EDTA blood samples in research was granted from the Royal Veterinary College Ethics and Welfare Committee (Approval number URN 2014 1292, 3rd September 2014).

Blood samples were obtained from laboratory Beagle dogs (*n*=90) enrolled as unvaccinated controls in one of four vaccination/challenge studies undertaken in Spain. As part of these studies, dogs were challenged with *L. infantum* (MCRI/ES/2006/BCN-720 MON 1) by IV injection of either amastigotes or promastigotes, according to the protocol shown in Supplementary Table 1. Dogs in studies A (*n*=30) and B (*n*=17) were challenged with 1×10⁶ promastigotes, dogs in study C (*n*=23) were challenged with 5×10⁷ promastigotes and dogs in study D (*n*=20) were challenged with 2×10⁶ amastigotes. Dogs were monitored for a period of up to 2 years and were regularly examined and subjected to diagnostic testing
during this period. Dogs were allocated a clinical score (0-2/2), for clinical parameters which included body condition, demeanour, skin lesions, mucous membrane colour, ocular lesions, lymph node size (Supplementary Table 2). A combined clinical score was then allocated for each time point, based on sum of the individual scores. Dogs were monitored for between 5 and 19 months and clinical scores were assigned every 2-8 weeks, depending on the study design.

Diagnostic testing

ELISA testing was performed to assess the presence of anti-
Leishmania antibodies as previously described with some modifications (Solano-Gallego et al., 2014). Briefly, 96-well flat-bottomed plates (Maxisorb, Nunc) were coated overnight at 4 °C with 100 µL per well of diluted sonicated crude L. infantum promastigotes (20 µg/ml; MCAN/ES/92/BCN-83/MON-1), then plates were emptied and left to dry at room temperature. Canine serum was diluted 1:200 in phosphate buffered saline (PBS) supplemented with 0.05% Tween 20 (PBST; Sigma Aldrich) containing 10 g/L dried skimmed milk (PBSTM) and 100 µL per well was added in duplicate. A calibrator sample was diluted 1:400 and then through 1:2 dilutions to 1:12800 to create a 6-point standard curve. Plates were incubated for 60 min at 37 °C, then washed thoroughly with PBST. Plates were incubated with 100 µL of sheep anti-dog IgG conjugated to horseradish peroxidase (Serotec) for 60 min at 37 °C. Absorbance was measured at 492 nm on an automatic ELISA reader (Thermoscientific MULTISKAN Spectrum). The results were expressed in ELISA units (EU) in relation to a known positive serum (used for calibration) and arbitrarily set at 100 EU. The positive cut-off was determined for each new antigen batch from the mean + 3 standard deviations (SD) for 50 serum samples from non-infected dogs collected before the study commenced. Serology was performed in three of the four studies, every 2-6 weeks, depending on study design.
Quantitative PCR was performed on bone marrow samples as previously described (Francino et al., 2006). Quantitative analysis was performed through absolute quantification from a 6-point standard curve, with serial dilutions of a parasite culture, top standard equivalent to 500 promastigotes, and values were expressed as genome copy/mL bone marrow aspirate. Testing for *L. infantum* DNA in bone marrow aspirates was performed in all dogs every 2-6 months.

Statistical analysis

Correlation between clinical score, ELISA and qPCR data was assessed using Kendall’s Tau correlation. Bias corrected and accelerated bootstrapping was performed to provide more robust 95% confidence intervals for this non-normally distributed data set, (1000 bootstrap samples were used except where indicated). Correlations were performed across all time points.

Each study group was analysed separately, since challenge and dose was likely to influence outcome. At each monthly time point studied and for each phenotype parameter (clinical score, serology and parasite load determined by qPCR), dogs were ranked based on whether they were positioned above or below the median score. Dogs consistently (>70% of time points in study) above the median score for each phenotype were categorised as high. Dogs consistently (>70% of time points in the study) below the median score for the phenotype were categorised as low. All other dogs were categorised as medium for each phenotype. Dogs that were euthanased as a result of *L. infantum* infection during the study period were assigned to the high category regardless of phenotyping method.
Haplotype frequencies were calculated and were compared between groups using Fisher’s exact test, with Bonferroni correction when all haplotypes were examined concurrently, in SPSS Statistics v22 (IBM, Hampshire, UK). Haplotype frequencies between different groups were compared for each phenotyping method. Each group was analysed separately and then all groups were analysed together.

**DLA genotyping**

Genomic DNA (gDNA) was extracted from EDTA blood samples using the GenElute™ Blood Genomic DNA Kit (Sigma-Aldrich), according to the manufacturer’s instructions. Polymerase chain reaction (PCR) was used to amplify DNA using DLA-specific primers. (2 μL at 20 pmol/μL final concentration; Sigma-Aldrich) were used. Primers used were DRB1 FOR CCGTCCCCACCAGCACATTTC, DRB1 M13 REV TGTAAAACGACGGCCAGTGTACACACCTCAGCACCA (adapted from Wagner et al., 1996b)); DQA1 M13 FOR TGTTAAGACGGCCAGTCTCTAGCTGACCATTTC, DQA1 REV GGACAGATTCAGTGAAGAGAG (adapted from Wagner et al., 1996a)); DQB1 M13 FOR TGTTAAGACGGCCAGTCTCTACTGCCCAGCCTC, DQB1 REV CACCTCGCCTGAACGTG (adapted from Wagner et al., 1998). Each reaction contained 5 μL Hi-Spec additive, 2.5 μL ImmoBuffer, 1.25 μL MgCl2 (2.5mM final concentration), 0.25 μL deoxynucleotide triphosphates (1 mM final concentration of all dNTPs) and 0.1 μL (1.25 IU) Immolase DNA polymerase (Bioline).

PCR was performed using a G-Storm GS1 Thermal Cycler (Gene Technologies). Reactions were heated to 95 °C for 10 min, followed by 35 cycles consisting of 94 °C for 40 s, 55 °C for 30 s for DQA1 or 60 °C for DRB1 and DQB1, and 72 °C for 1 min, with a final extension step at 72 °C for 10 min. PCR products were processed using the GenElute PCR
Clean-up Kit (Sigma-Aldrich) and submitted for sequencing (Source Bioscience) using M13F primer.

Sequencing results were analysed using CLC Workbench v 6.9.1 (CLC bio). DLA alleles were assigned using SBT Engine 3.6.1 software (GenDx). Three locus haplotypes were assembled from the assigned alleles, based on previous data regarding common haplotypes in Beagle dogs (Soutter et al., 2015).

**Results**

*Clinical scoring*

Examination of clinical score data revealed variability in clinical signs and disease severity between individual dogs that had received the same infection dose and type. There was a variable clinical picture over time, with most dogs having higher clinical scores towards the end of the study period (a representative example of clinical scores for some individual dogs in study B is shown in Fig. 1). In all groups, some individuals began to develop clinical signs relatively quickly; with enlargement of peripheral lymph nodes 2-6 weeks post-infection. Skin lesions appeared from 2 months post-infection and evidence of multi-systemic disease and higher clinical scores from 7 months onwards in some individuals. Two groups (C and D) were monitored for 6 and 5 months post-infection, respectively, and more severe or multi-systemic clinical signs were not observed in these groups.

*Serology*

All dogs were seropositive for *Leishmania*-specific antibodies by the end of their respective study periods in the three studies that included serological testing. *Leishmania*-
specific antibodies increased over time after infection in all studies. *Leishmania* antibody reactivity varied between dogs receiving the same challenge and dose at the same time post-infection (a representative example of serology results for some individual dogs [study B] is shown in Fig. 2).

*Parasite load*

Almost all dogs were qPCR positive for *Leishmania* DNA in bone marrow by the end of their respective study periods. Most dogs that had received IV amastigote challenge were qPCR positive in the bone marrow at the first time point where this was assessed (2 months after infection). When quantified, *Leishmania* DNA concentration in the bone marrow was highly variable between individuals, even within the same study at the same time point (a representative example of test results for some individual dogs [study B] is shown in Fig. 3). While *Leishmania* DNA in the bone marrow tended to increase over time for each group as a whole, some dogs within the group did not demonstrate a change in parasite load in the bone marrow over the study period.

*Categorisation of dogs based on outcome of infection*

Clinical scores, serology test results and parasite load estimation by qPCR failed to show any consistent relationships, despite expectations of a positive association (i.e. sick dogs showing high clinical scores, strong seropositivity and high parasite loads and the opposite for resistant dogs). In Studies B and C, parasite DNA concentration in the bone marrow significantly correlated with *Leishmania*-specific antibodies (*P*<0.05), although no such correlation was demonstrated in study D (Supplementary Fig. 1). Clinical score did not correlate with *Leishmania*-specific antibodies nor qPCR test results in any of the study groups, except for Study B, where clinical score correlated inversely with *Leishmania* DNA
concentration in the bone marrow ($\tau=-.252$, BCa CI [-.396, -.061], $P=0.013$; Supplementary Figs. 2 and 3). Therefore, we could not categorise dogs into susceptible vs. resistant phenotypes based on combining the available data for each individual dog. Because the study population consisted of four different challenge trials, each with a different protocol, it was not considered appropriate to combine the dogs into a single cohort. Therefore, it was decided to undertake further analysis separately for each study group and to phenotype the dogs independently, according to clinical score, serology or parasite load (Table 1).

**DLA typing**

We identified 10 different DLA haplotypes (found in two or more dogs) in this study, plus three other haplotypes found in single dogs only, with substantial variation in the DLA profile between study groups (Table 2). Two haplotypes were common between all four study groups, while four haplotypes were only present in one of the four groups. The most common haplotype was DLA-DRB1*006:01--DQA1*005:01:1--DQB1*007:01. Two DQB1 alleles were amplified as part of the DLA-DRB1*019:01--DQA1*004:01-- DQB1*013:03/017:01 haplotype. We could not ascertain whether both of these DQB1 alleles are expressed as mRNA was not available. A total of 10 homozygous dogs were observed, with between 2-3 homozygous dogs in each group.

**DLA association with clinical score**

The DLA allele or haplotype frequencies did not differ between groups based on clinical score in any of the individual studies (Supplementary Data Table 3), or when all four study groups were combined ($\chi^2=22.91$, df=26, $P=0.64$).

**DLA association with Leishmania serology**
When we compared DLA haplotype frequencies between categories based on serology test results, DLA haplotype frequencies differed between groups only in Study C (Table 3). Specifically, the DLA-DRB1*001:02--DQA1*001:01--DQB1*002:01 haplotype was more common in the high seropositivity group than the low seropositivity group \((P=0.03, \text{OR}=15.4, 95\% \text{CI}=1.5-170.0)\) or the medium seropositivity group \((P=0.025, \text{OR}=6.3, 95\% \text{CI}=1.3-30.5)\), but frequency of this haplotype did not differ between medium and low seropositivity groups \((P>0.05)\). The DLA-DRB1*006:01--DQA1*005:01--DQB1*007:01 haplotype was more common in the medium seropositivity group than the high seropositivity group \((P=0.013)\). Haplotype frequencies did not differ between groups in studies B or D \((P>0.05; \text{Supplementary Table 4})\). Similarly, haplotype frequency between groups did not differ when all groups were combined \((\chi^2=19.03, \text{df}=22, P=0.65)\).

There was no association with DLA-DRB1 or DLA-DQA1 and seropositivity in any of the groups studied. However, DLA-DQB1 allele 002:01 in study C was more common in the high seropositivity group than the medium seropositivity group \((P=0.01, \text{odds ratio [OR]} =8.0, 95\% \text{CI}=1.6-40.0)\) or the low seropositivity group \((P=0.01, \text{OR}=15.0, 95\% \text{CI}=2.02-111.2)\). Conversely DLA-DQB1 allele 007:01 in study C was more common in the medium group than the high group \((P=0.01, \text{OR}=\infty)\).

**DLA association with Leishmania DNA concentration in the bone marrow**

When we compared DLA haplotype frequencies between categories based on parasite DNA concentration in the bone marrow, the DLA-DRB1*006:01--DQA1*005:01:1--DQB1*007:01 haplotype was more common in the medium group than the low group \((P=0.02, \text{OR}=22.0, 95\% \text{CI}=1.5-314.3)\) only in study B (Table 4), but no other differences existed (Supplementary Table 5). Haplotype frequency between groups did not differ when all studies were combined \((\chi^2=30.16, \text{df}=26, P=0.26)\).
There was no association with DLA-DRB1 or DLA-DQB1 and parasite load in any of the groups studied. However, DLA-DQA1 allele 001:01 was more common in the medium group than the low group in Study C ($P=0.01$, OR=10.0, 95%CI=1.76-56.9).

Discussion

We examined the relationship between variability in disease expression following experimental infection with *L. infantum* in unvaccinated Beagles used in vaccine studies and DLA haplotypes frequencies, to determine whether these immune response genes impacted the clinical outcome of infection. Clinical scores and the clinical infection course were highly variable between dogs, as were serological responses and parasite detection in the bone marrow, although all dogs seroconverted and most dogs became qPCR positive. As clinical score did not consistently correlate with the serological response or parasite load, each parameter was used separately to categorise dogs according to disease expression. We found a potential association between DLA haplotype and serological response to experimental infection with *L. infantum* in Beagles, although this was not found in all groups. There was no association between DLA haplotype and clinical disease expression after infection.

All dogs in the study were experimentally infected with *L. infantum* by IV injection, which is not necessarily comparable with natural infection by biting sandflies. Additionally, the infective dose of either promastigotes or amastigotes ($1\times10^6$ parasites and $2\times10^8$ parasites, respectively) was much greater than in natural infection, which researchers believe to be approximately 100-1000 parasites per bite (Saridomichelakis, 2009; Rogers, 2012). In IV infection models, researchers believe that parasite dissemination to the organs and
therefore clinical signs occur more quickly (Moreno and Alvar, 2002), which means that studies are less time consuming, although not truly representative of natural infection. However, since the dogs are kept in a controlled environment and are exposed to a fixed dose of parasite at a known time point, there are less random effects that could influence the outcome of infection, which is crucial in providing efficacy data for vaccine licencing and potentially useful in evaluating potential genetic factors associated with disease.

Clinical scores were used to evaluate the nature and severity of clinical signs at different time points following IV infection with *L. infantum*. Most dogs developed some degree of illness during the study and some dogs started to develop clinical signs at 2-3 months post-infection, although more severe clinical signs were not evident until around 7 months post-infection. Our findings mirror those reported in other studies using IV infection methods, where clinical signs often appear from around 2-4 months post-infection (Carrera et al., 1996; Poot et al., 2005) and clinical outcomes of infection vary between dogs (Nieto et al., 1999; Campino et al., 2000).

We used clinical scores to quantify the clinical signs observed in dogs over time and to distinguish very sick dogs from those with mild clinical signs. However, dogs with moderate clinical scores were more difficult to evaluate, particularly in the absence of clinicopathological data. Such data could have enabled us to use a clinical staging system such as the Leishvet guidelines or those proposed by the Canine Leishmaniasis Working Group (Solano-Gallego et al., 2009; Roura et al., 2013), which stage dogs based on clinical signs, clinicopathological abnormalities and *Leishmania* diagnostic testing information. However, clinical staging might not distinguish dogs predisposed to developing clinical disease from those that are more resistant to infection, when dogs are exposed to a high dose.
of pathogen in experimental infection and thus might not have been useful in phenotyping dogs.

High Leishmania antibody reactivity accompanied by clinical and clinicopathological abnormalities is considered diagnostic for canine leishmaniosis (Solano-Gallego et al., 2011). Leishmania-specific antibody levels tended to increase over time regardless of the challenge type. Previous studies have demonstrated that seroconversion following infection is variable with regards to the number of dogs that seroconvert and the time of seroconversion post-infection with promastigote challenge (Nieto et al., 1999; Campino et al., 2000; Paranhos-Silva et al., 2003). Assessment of cell mediated immunity was not performed as the relationship between T-helper phenotypes and disease outcome is still unclear (Maia and Campino, 2012) and thus could have further confounded efforts to categorise dogs based on outcome of infection.

Quantitative PCR for detection of Leishmania DNA was used to confirm the presence of active infection and bone marrow aspirates have greater sensitivity for detection of parasite DNA by qPCR compared with peripheral blood samples (Francino et al., 2006; Hernandez et al., 2015). Most dogs in our study were qPCR positive in the bone marrow by the end of the study, similar to previously published studies (Leandro et al., 2001; Fernandez-Cotrina et al., 2013).

Dogs in our study displayed variability for all measured parameters, even those dogs that had received the same type of challenge, dose and were assessed at the same time after infection. This might reflect differences in individual dogs’ immune responses to L. infantum and the ability to control parasite numbers and prevent disease. In this study, the clinical
score did not appear to correlate with serology test results or with parasite DNA detection in the bone marrow and, unexpectedly and counter-intuitively, in one study the clinical score was inversely correlated with parasite load in the bone marrow. We did not anticipate this result, as several studies have indicated that dogs with demonstrable clinical signs following natural infection had elevated parasite loads in various tissues, as well as high antibody reactivity (Reis et al., 2006; Dos-Santos et al., 2008; Solano-Gallego et al., 2016). Similarly, *Leishmania*-specific antibody positivity have been shown to correlate with parasite loads in the tissues of dogs naturally infected with *L. infantum* (Reis et al., 2006; Manna et al., 2009; Solano-Gallego et al., 2016). It is possible that the experimental model using Beagle dogs resulted in a more homogenous clinical picture than one might expect with natural infection in a more outbred population. Furthermore, it is possible that some dogs would have developed more severe clinical signs or multi-systemic clinical signs if they had been followed for a longer period of time. It is also possible that clinical score does not adequately separate dogs that are somewhere in the middle of the disease spectrum and that this could be having an impact on lack of correlation between parameters.

The individual study designs were somewhat variable, which meant that phenotyping dogs for a genetic association study was challenging and groups could not be easily combined. Additionally, as clinical disease was not consistently associated with anti-*Leishmania* antibodies or with parasite load in the bone marrow, we could not categorise dogs, based on both clinical disease, immunological parameters and infection status, so we analysed the relationships of these parameters independently. Alternative phenotyping methods could have assessed *Leishmania*-specific antibodies or *Leishmania* DNA concentration as a quantitative trait, or used all diagnostic parameters in a mixed model. However, as the samples and diagnostic data were obtained from several different studies,
there was variability in the time points when these parameters were measured, which would
have been difficult to model. The phenotyping method used, while far from perfect, aimed to
assess disease expression of each dog compared against the median disease expression of the
group at each time point. This method could then be used to distinguish individual dogs with
disease expression consistently higher or lower than the group median and could therefore be
considered to be highly susceptible or less susceptible for each parameter measured. All
genetic association studies were likely to be underpowered as a result of the small sample
size in each group and should be repeated in a larger cohort.

Ten DLA haplotypes were identified in at least three Beagles. While this degree of
restriction at the DLA locus is not as marked compared with some breeds, such as the
Dobermann and the Rottweiler (Kennedy et al., 2002), it could affect the repertoire of peptide
epitopes that can be presented to CD4+ T lymphocytes upon antigenic stimulation. This
limited diversity could also influence the response to experimental infection with *L. infantum*
and vaccine responses such that a product deemed safe and efficacious in Beagles would not
necessarily perform similarly in other dog breeds. Furthermore, there was substantial
variation in DLA profile between the different study groups, which indicates that
immunogenetic profile has not been a factor in selection of dogs for these vaccine studies.
However, differences in DLA profile between studies and within a study could result in
variability in response to infection and vaccination between groups that is undesirable when
trying to determine the efficacy of a new vaccine.

None of the study groups differed in DLA haplotype frequencies when assessed
against clinical outcomes. One group showed a difference in haplotype frequencies between
different serological responses. Specifically, the DLA-DRB1*001:02--DQA1*001:01--
DQB1*002:01 haplotype was associated with a sustained elevation of anti-*Leishmania* antibodies over the study period, compared with dogs that had low or fluctuating antibody reactivity over the same period. A previous study examining DLA haplotypes in crossbreed dogs in a *Leishmania* endemic region naturally infected with *Leishmania* reported that the DRB1 genotype 015:02 was associated with increased anti-*Leishmania* antibody levels and PCR positivity in the bone marrow, but did not demonstrate an association with clinical score (Quinnell et al., 2003). This particular DRB1 allele was not present in the Beagle population and therefore could not be assessed. Similarly, the DRB1*001:02 allele found to differ between groups in our study was not one of the common alleles described in a study of natural infection of Brazilian dogs. Interestingly, the haplotype DLA-DRB1*015:01--DQA1*009:01--DQB1*001:01, was found in dogs with high sustained parasite load in the bone marrow, but did not differ in frequency from those showing low parasite loads ($P=0.06$). It is worth noting that DLA-DRB1*015:01 differs by a single amino acid from 015:02 as a result of a single base pair substitution (Kennedy et al., 1998). Both our study and a previously published study examining naturally infected dogs (Quinnell et al., 2003) found a DLA association with the antibody response, but not with clinical disease expression. DLA genes might influence antigen presentation and impact on the subsequent antibody response to *L. infantum*, but it is possible that there are other host and environmental factors that determine disease expression. It is also possible that the clinical scoring system used is not a sufficiently robust method for distinguishing highly sick individual dogs from those with less severe clinical signs.

In one group, dogs of another haplotype (DLA-DRB1*006:01--DQA1*005:01:1--DQB1*007:01) that demonstrated intermediate anti-*Leishmania* seropositivity occurred more
frequently than in those with high seropositivity. However, in another group, the DLA-DRB1*006:01--DQA1*005:01:1--DQB1*007:01 haplotype occurred more frequently in dogs with an intermediate parasite DNA concentration than in those showing low parasite DNA concentration in the bone marrow over the study period. It is possible that the effect of homozygosity for DLA-DRB1*006:01--DQA1*005:01:1--DQB1*007:01 could be creating a spurious result for this particular haplotype. Therefore, we repeated this analysis using number of dogs rather than number of haplotypes (data not shown) and there was not a significant association with seropositivity, or with parasite load ($P > 0.05$; Supplementary Tables 6 and 7).

Further work is required in a larger group of dogs receiving a similar experimental challenge of *L. infantum* or in a naturally infected cohort to identify if a true association between DLA genes and outcome of infection with *L. infantum* exists in dogs. Examination of other immune response genes is also warranted and would expand existing knowledge on disease pathogenesis following infection and potentially identify new vaccine targets. The selection of dogs for vaccine studies based on their genetic susceptibility profile might result in less individual variability and more robust trials. Furthermore, early identification of dogs more predisposed to developing clinical disease would facilitate targeted prevention and vaccination strategies in endemic regions.

**Conclusions**

Beagle dogs demonstrated variability in their clinical and serological response to experimental infection with *L. infantum* and in their ability to control parasite load in the tissues, despite some degree of restriction in DLA diversity. No DLA association with clinical score was observed. Investigation of the effect of DLA haplotypes on the outcome of
experimental infection revealed a potential haplotype (DRB1*001:02--DQA1*001:01--
DQB1*002:01) which was associated with strong serological responses, which are normally
counterproductive in this type of intracellular infection, although such an association could
not be demonstrated in all groups. Immunogenetic investigation of larger cohorts of dogs,
following a standardised experimental challenge is warranted. Immunogenetic analysis could
inform vaccine study design and appropriate selection of dogs for such trials, so that vaccine
efficacy studies can be more economical and ethical, with a reduction in the number of dogs
used.

Conflict of interest statement

This research was sponsored by Zoetis, which supplied the samples for genetic
analysis and provided data for phenotyping the dogs in this study. Zoetis played no role in in
the analysis and interpretation of data, nor in the decision to submit the manuscript for
publication. Susanna Martorell is an employee of Zoetis. None of the other authors of this
paper has a financial or personal relationship with other people or organisations that could
inappropriately influence or bias the content of the paper.

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(BB/I015655/1) in partnership with Zoetis.

Appendix: Supplementary data
Supplementary data associated with this article can be found, in the online version, at doi: ...'

References


Table 1.
Number of dogs categorised by their disease phenotype according to phenotyping method

<table>
<thead>
<tr>
<th>Phenotyping method</th>
<th>Disease phenotype</th>
<th>Number of dogs (% of group)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Study A (n=30)</td>
<td>Study B (n=17)</td>
</tr>
<tr>
<td>Clinical score</td>
<td>High</td>
<td>17 (57%)</td>
</tr>
<tr>
<td></td>
<td>Medium</td>
<td>8 (27%)</td>
</tr>
<tr>
<td></td>
<td>Low</td>
<td>5 (16%)</td>
</tr>
<tr>
<td>Serology</td>
<td>High</td>
<td>a</td>
</tr>
<tr>
<td></td>
<td>Medium</td>
<td>a</td>
</tr>
<tr>
<td></td>
<td>Low</td>
<td>a</td>
</tr>
<tr>
<td>Parasite load (BM)</td>
<td>High</td>
<td>19 (63%)</td>
</tr>
<tr>
<td></td>
<td>Medium</td>
<td>9 (30%)</td>
</tr>
<tr>
<td></td>
<td>Low</td>
<td>2 (7%)</td>
</tr>
</tbody>
</table>

*Serology not performed in this study*
Table 2.
DLA haplotypes identified in Beagles experimentally infected with *L. infantum*.

<table>
<thead>
<tr>
<th>Haplotype</th>
<th>Experimental infection study (number of haplotypes)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DRB1</td>
</tr>
<tr>
<td>001:01</td>
<td>001:01</td>
</tr>
<tr>
<td>001:02</td>
<td>001:01</td>
</tr>
<tr>
<td>002:01</td>
<td>009:01</td>
</tr>
<tr>
<td>006:01</td>
<td>001:01</td>
</tr>
<tr>
<td>006:01</td>
<td>001:01</td>
</tr>
<tr>
<td>006:01</td>
<td>005:01:1</td>
</tr>
<tr>
<td>008:01</td>
<td>003:01</td>
</tr>
<tr>
<td>014:01</td>
<td>001:01</td>
</tr>
<tr>
<td>015:01</td>
<td>009:01</td>
</tr>
<tr>
<td>019:01</td>
<td>004:01</td>
</tr>
<tr>
<td>Other single haplotypes</td>
<td>2</td>
</tr>
<tr>
<td>Total (haplotypes)</td>
<td>60</td>
</tr>
<tr>
<td>Total number of dogs</td>
<td>30</td>
</tr>
<tr>
<td>Total number of homozygous dogs</td>
<td>2</td>
</tr>
</tbody>
</table>

<sup>a</sup> One homozygous dog with this haplotype
<sup>b</sup> Three homozygous dogs with this haplotype
### Table 3.

**DLA haplotypes in Study C phenotyped according to serology test results**

<table>
<thead>
<tr>
<th>Haplotype</th>
<th>Number of haplotypes</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DRB1</td>
<td>DQA1</td>
<td>DQB1</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>High</td>
</tr>
<tr>
<td>001:01</td>
<td>001:01</td>
<td>002:01</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>001:02</td>
<td>001:01</td>
<td>002:01</td>
<td>7&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4</td>
</tr>
<tr>
<td>006:01</td>
<td>001:01</td>
<td>008:02</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>006:01</td>
<td>005:01</td>
<td>007:01</td>
<td>0</td>
<td>9&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>008:01</td>
<td>003:01</td>
<td>004:01</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>014:01</td>
<td>001:01</td>
<td>008:01</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>015:01</td>
<td>009:01</td>
<td>001:01</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>019:01</td>
<td>004:01</td>
<td>013:03/017:01</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Total (haplotypes)</td>
<td>12</td>
<td>22</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>Total (dogs)</td>
<td>6</td>
<td>11</td>
<td>6</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Dogs with *Leishmania*-specific IgG (ELISA Units) above the median in 70% of the study time points are categorised as high susceptibility. Dogs with *Leishmania*-specific IgG levels below the median in 70% of the study time points are categorised as low susceptibility. Each haplotype is made up of three loci- DLA-DRB1, DLA-DQA1 and DLA-DQB1.  

<sup>b</sup> One dog homozygous for this haplotype  

<sup>c</sup> *P*<0.05
**Table 4.**
DLA haplotypes in Study B phenotyped according to *Leishmania* DNA concentration in the bone marrow

<table>
<thead>
<tr>
<th>Haplotype</th>
<th>Number of haplotypes</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>DRB1</td>
<td>DQA1</td>
<td>DQB1</td>
</tr>
<tr>
<td>001:01</td>
<td>001:01</td>
<td>002:01</td>
</tr>
<tr>
<td>002:01</td>
<td>009:01</td>
<td>001:01</td>
</tr>
<tr>
<td>006:01</td>
<td>001:01</td>
<td>008:02</td>
</tr>
<tr>
<td>006:01</td>
<td>001:01</td>
<td>008:011</td>
</tr>
<tr>
<td>006:01</td>
<td>005:01:1</td>
<td>007:01</td>
</tr>
<tr>
<td>008:01</td>
<td>003:01</td>
<td>004:01</td>
</tr>
<tr>
<td>014:01</td>
<td>001:01</td>
<td>008:01:1</td>
</tr>
<tr>
<td>Other single haplotypes</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Total (haplotypes)</td>
<td>16</td>
<td>6</td>
</tr>
<tr>
<td>Total (dogs)</td>
<td>8</td>
<td>3</td>
</tr>
</tbody>
</table>

N/A, Not applicable

*a* Dogs with *Leishmania* DNA concentration (genome copy/mL) in the bone marrow above the median in 70% of the study time points are categorised as high susceptibility. Dogs with *Leishmania* DNA concentration in the bone marrow below the median in 70% of the study time points are categorised as low susceptibility. Each haplotype is made up of three loci—DLA-DRB1, DLA_DQA1 and DLA-DQB1. $P$ values comparing all three groups were determined by the Fisher’s exact probability test.

*b* One dog homozygous for this haplotype

*c* $P<0.05$
Figure legends

Fig. 1. Clinical score over time for representative dogs in study B. Dogs in the high category had scores above the median in >70% of the time points. Dogs in the low category had scores below the median in >70% of the time points.
Fig. 2. Serology results for representative dogs in study B. *Leishmania*-specific IgG was measured by ELISA. Dogs in the high category had scores above the median in >70% of the time points. Dogs in the low category had scores below the median in >70% of the time points.
Fig. 3. Parasite load in the bone marrow for representative dogs in study B. *Leishmania* DNA in the bone marrow was measured by qPCR. The median score for each time point is represented by a horizontal line. Dogs in the high category had scores above the median in >70% of the time points. Dogs in the low category had scores below the median in >70% of the time points.