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1 **Original Article**
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3 **Inconsistent MHC Class II association in Beagles experimentally infected with**
4 ***Leishmania infantum***
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20 **Abstract**

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

31

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

40

41 *Keywords:* Beagle; DLA; Genetics; Leishmaniosis; MHC

42 **Introduction**

43 The protozoan parasite *Leishmania infantum* is most commonly transmitted between
44 mammalian hosts via biting female sandflies, belonging to the genera *Phlebotomus* or
45 *Lutzomyia*. The distribution of such vectors largely limits infection to particular geographical
46 regions (Killick-Kendrick, 1999). *Leishmania infantum* is endemic in the Mediterranean
47 basin, Central and South America and parts of Asia and Africa (Palatnik-de-Sousa and Day,
48 2011). Europe has approximately 2.5 million infected dogs, based on seroprevalence data,
49 (Moreno and Alvar, 2002) although no official surveillance system for recording the number
50 of dogs infected exists.
51
52 Canine leishmaniosis presents with diverse clinicopathological abnormalities and
53 clinical outcomes. Furthermore, there appears to be significant individual variation following
54 infection with the *L. infantum* parasite with only some dogs developing clinical disease
55 (Baneth et al., 2008). In an experimental infection model utilised in vaccine studies, high
56 doses of amastigotes or promastigotes are given IV, but even under these circumstances,
57 some dogs do not develop clinical signs over the study period, despite a relatively large
58 challenge dose (Campino et al., 2000; Costa et al., 2013).
59
60 The non-specific clinical signs and the absence of a reference standard test complicate
61 the diagnosis of canine leishmaniosis (Rodriguez-Cortes et al., 2010). Serological testing is
62 often used for diagnostic purposes, to monitor the infection course and/or the response to
63 treatment. However, while *Leishmania*-specific antibody levels do not correlate with disease
64 protection, high antibody reactivity is associated with clinical disease (Reis et al., 2006).
65 Detection of *Leishmania* DNA in the tissues with PCR is a sensitive alternative technique for

66 identifying infection (Cortes et al., 2004) and high parasite load in the tissues is associated
67 with clinical disease (Dos-Santos et al., 2008).
68
69 Previous canine studies appeared to confirm a role for T-cell mediated immunity
70 (CMI) in resistance to canine leishmaniosis, with IFN-γ, produced by stimulated lymphocytes
71 from subclinically infected dogs, able to lyse *Leishmania* infected macrophages, in contrast
72 with lymphocytes from clinically infected dogs (Pinelli et al., 1995). Despite several studies
73 examining cell mediated immunity in dogs, a clear picture of the T-helper phenotypes
74 associated with disease outcome has not emerged and results are often contradictory (Hosein
75 et al., 2017). Therefore, CMI assays are infrequently performed to diagnose clinical
76 leishmaniosis and their utility for predicting outcome of infection is not always reliable.
77
78 The genetic background of the host might play a role in determining the outcome of
79 infection with *L. infantum* and differences in susceptibility between different dog breeds has
80 been suggested. The Ibizan hound in particular has been identified as a potentially resistant
81 breed (Solano-Gallego et al., 2000). Other studies have suggested that the Cocker spaniel
82 and Boxer breeds might be more at risk of developing clinical disease (Franca-Silva et al.,
83 2003). As the outcome to *L. infantum* infection is largely dependent on the host immune
84 response, much of the genetic research has focussed on immune response genes that might
85 determine the outcome of infection.
86
87 Dog leukocyte antigen (DLA) class II genes determine antigen presentation by MHC
88 class II molecules and influence the subsequent immune response; therefore, they might also
89 determine the ability to control *L. infantum* parasite numbers in tissues and clinical outcome.
90 A previous study has examined DLA genes in a naturally infected group of cross breed dogs

91 in Brazil and DLA-DRB1 015:02 was associated with increased risk of *L. infantum* infection
92 (Quinnell et al., 2003). The impact of the DLA background of laboratory Beagle dogs on the
93 response to experimental infection, undertaken as part of vaccine efficacy studies, has not
94 been examined and immunogenetic profiling of dogs enrolled in vaccine challenge studies
95 might provide valuable additional information in terms of the response to vaccination and the
96 clinical outcome following experimental infection. In this study, DLA class II genes were
97 examined in four groups of Beagle dogs experimentally challenged with *L. infantum* and
98 studied post-challenge as part of vaccine studies.
99
100 **Materials and methods**
101 *Study population*
102 EDTA blood samples were taken as part of ongoing commercial studies into candidate
103 vaccines. Blood sampling was undertaken by appropriately trained Zoetis staff in accordance
104 with the relevant regulatory approval at the partner institution. Approval for use of residual
105 EDTA blood samples in research was granted from the Royal Veterinary College Ethics and
106 Welfare Committee (Approval number URN 2014 1292, 3rd September 2014).
107
108 Blood samples were obtained from laboratory Beagle dogs (*n*=90) enrolled as
109 unvaccinated controls in one of four vaccination/challenge studies undertaken in Spain. As
110 part of these studies, dogs were challenged with *L. infantum* (MCRI/ES/2006/BCN-720
111 MON 1) by IV injection of either amastigotes or promastigotes, according to the protocol
112 shown in Supplementary Table 1. Dogs in studies A (*n*=30) and B (*n*=17) were challenged
113 with 1 x10^6 promastigotes, dogs in study C (*n*=23) were challenged with 5 x10^7 promastigotes
114 and dogs in study D (*n*=20) were challenged with 2 x10^8 amastigotes. Dogs were monitored
115 for a period of up to 2 years and were regularly examined and subjected to diagnostic testing

116 during this period. Dogs were allocated a clinical score (0-2/2), for clinical parameters which
117 included body condition, demeanour, skin lesions, mucous membrane colour, ocular lesions,
118 lymph node size (Supplementary Table 2). A combined clinical score was then allocated for
119 each time point, based on sum of the individual scores. Dogs were monitored for between 5
120 and 19 months and clinical scores were assigned every 2-8 weeks, depending on the study
121 design.
122
123 *Diagnostic testing*
124 ELISA testing was performed to assess the presence of anti-*Leishmania* antibodies as
125 previously described with some modifications (Solano-Gallego et al., 2014). Briefly, 96-well
126 flat-bottomed plates (Maxisorb, Nunc) were coated overnight at 4 °C with 100 µL per well of
127 diluted sonicated crude *L. infantum* promastigotes (20 µg/ml; MCAN/ES/92/BCN-83/MON-
128 1), then plates were emptied and left to dry at room temperature. Canine serum was diluted
129 1:200 in phosphate buffered saline (PBS) supplemented with 0.05% Tween 20 (PBST; Sigma
130 Aldrich) containing 10 g/L dried skimmed milk (PBSTM) and 100 µL per well was added in
131 duplicate. A calibrator sample was diluted 1:400 and then through 1:2 dilutions to 1: 12800
132 to create a 6-point standard curve. Plates were incubated for 60 min at 37 °C, then washed
133 thoroughly with PBST. Plates were incubated with 100 µL of sheep anti-dog IgG conjugated
134 to horseradish peroxidase (Serotec) for 60 min at 37 °C. Absorbance was measured at 492
135 nm on an automatic ELISA reader (Thermoscientific MULTISKAN Spectrum). The results
136 were expressed in ELISA units (EU) in relation to a known positive serum (used for
137 calibration) and arbitrarily set at 100 EU. The positive cut-off was determined for each new
138 antigen batch from the mean + 3 standard deviations (SD) for 50 serum samples from non-
139 infected dogs collected before the study commenced. Serology was performed in three of the
140 four studies, every 2-6 weeks, depending on study design.

141

142 Quantitative PCR was performed on bone marrow samples as previously described

143 (Francino et al., 2006). Quantitative analysis was performed through absolute quantification

144 from a 6-point standard curve, with serial dilutions of a parasite culture, top standard

145 equivalent to 500 promastigotes, and values were expressed as genome copy/mL bone

146 marrow aspirate. Testing for *L. infantum* DNA in bone marrow aspirates was performed in

147 all dogs every 2-6 months.

148

149 *Statistical analysis*

150 Correlation between clinical score, ELISA and qPCR data was assessed using

151 Kendall's Tau correlation. Bias corrected and accelerated bootstrapping was performed to

152 provide more robust 95% confidence intervals for this non-normally distributed data set,

153 (1000 bootstrap samples were used except where indicated). Correlations were performed

154 across all time points.

155

156 Each study group was analysed separately, since challenge and dose was likely to

157 influence outcome. At each monthly time point studied and for each phenotype parameter

158 (clinical score, serology and parasite load determined by qPCR), dogs were ranked based on

159 whether they were positioned above or below the median score. Dogs consistently (>70% of

160 time points in study) above the median score for each phenotype were categorised as high.

161 Dogs consistently (>70% of time points in the study) below the median score for the

162 phenotype were categorised as low. All other dogs were categorised as medium for each

163 phenotype. Dogs that were euthanased as a result of *L. infantum* infection during the study

164 period were assigned to the high category regardless of phenotyping method.

165

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^{TM} 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 TGTAAAACGACGGCCAGTGTCACACACCTCAGCACCA (adapted from (Wagner et al., 1996b)); DQA1 M13 FOR TGTAAAACGACGGCCAGTCTCAGCTGACCATGTTGC, DQA1 REV GGACAGATTCAGTGAAGAGAG (adapted from (Wagner et al., 1996a)); DQB1 M13 FOR TGTAAAACGACGGCCAGTCTCACTGGCCCGGCCTGTCTC, DQB1 REV CACCTCGCCGCTGAACGTG (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

191 Clean-up Kit (Sigma-Aldrich) and submitted for sequencing (Source Bioscience) using M13F
192 primer.
193
194 Sequencing results were analysed using CLC Workbench v 6.9.1 (CLC bio). DLA
195 alleles were assigned using SBT Engine 3.6.1 software (GenDx). Three locus haplotypes
196 were assembled from the assigned alleles, based on previous data regarding common
197 haplotypes in Beagle dogs (Soutter et al., 2015).
198
199 **Results**
200 *Clinical scoring*
201 Examination of clinical score data revealed variability in clinical signs and disease
202 severity between individual dogs that had received the same infection dose and type. There
203 was a variable clinical picture over time, with most dogs having higher clinical scores
204 towards the end of the study period (a representative example of clinical scores for some
205 individual dogs in study B is shown in Fig. 1). In all groups, some individuals began to
206 develop clinical signs relatively quickly; with enlargement of peripheral lymph nodes 2-6
207 weeks post-infection. Skin lesions appeared from 2 months post-infection and evidence of
208 multi-systemic disease and higher clinical scores from 7 months onwards in some
209 individuals. Two groups (C and D) were monitored for 6 and 5 months post-infection,
210 respectively, and more severe or multi-systemic clinical signs were not observed in these
211 groups.
212
213 *Serology*
214 All dogs were seropositive for *Leishmani*a-specific antibodies by the end of their
215 respective study periods in the three studies that included serological testing. *Leishmania-*

216 specific antibodies increased over time after infection in all studies. *Leishmania* antibody
217 reactivity varied between dogs receiving the same challenge and dose at the same time post-
218 infection (a representative example of serology results for some individual dogs [study B] is
219 shown in Fig. 2).
220
221 *Parasite load*
222 Almost all dogs were qPCR positive for *Leishmania* DNA in bone marrow by the end
223 of their respective study periods. Most dogs that had received IV amastigote challenge were
224 qPCR positive in the bone marrow at the first time point where this was assessed (2 months
225 after infection). When quantified, *Leishmania* DNA concentration in the bone marrow was
226 highly variable between individuals, even within the same study at the same time point (a
227 representative example of test results for some individual dogs [study B] is shown in Fig. 3).
228 While *Leishmania* DNA in the bone marrow tended to increase over time for each group as a
229 whole, some dogs within the group did not demonstrate a change in parasite load in the bone
230 marrow over the study period.
231
232 *Categorisation of dogs based on outcome of infection*
233 Clinical scores, serology test results and parasite load estimation by qPCR failed to
234 show any consistent relationships, despite expectations of a positive association (i.e. sick
235 dogs showing high clinical scores, strong seropositivity and high parasite loads and the
236 opposite for resistant dogs). In Studies B and C, parasite DNA concentration in the bone
237 marrow significantly correlated with *Leishmania*-specific antibodies ($P<0.05$), although no
238 such correlation was demonstrated in study D (Supplementary Fig. 1). Clinical score did not
239 correlate with *Leishmania*-specific antibodies nor qPCR test results in any of the study
240 groups, except for Study B, where clinical score correlated inversely with *Leishmania* DNA

241 concentration in the bone marrow (τ= -.252, BCa CI [-.396,-.061], *P*=0.013; Supplementary
242 Figs. 2 and 3). Therefore, we could not categorise dogs into susceptible vs. resistant
243 phenotypes based on combining the available data for each individual dog. Because the
244 study population consisted of four different challenge trials, each with a different protocol, it
245 was not considered appropriate to combine the dogs into a single cohort. Therefore, it was
246 decided to undertake further analysis separately for each study group and to phenotype the
247 dogs independently, according to clinical score, serology or parasite load (Table 1).
248
249 *DLA typing*
250 We identified 10 different DLA haplotypes (found in two or more dogs) in this study,
251 plus three other haplotypes found in single dogs only, with substantial variation in the DLA
252 profile between study groups (Table 2). Two haplotypes were common between all four study
253 groups, while four haplotypes were only present in one of the four groups. The most
254 common haplotype was DLA-DRB1*006:01--DQA1*005:01:1--DQB1*007:01. Two DQB1
255 alleles were amplified as part of the DLA-DRB1*019:01--DQA1*004:01--
256 DQB1*013:03/017:01 haplotype. We could not ascertain whether both of these DQB1
257 alleles are expressed as mRNA was not available. A total of 10 homozygous dogs were
258 observed, with between 2-3 homozygous dogs in each group.
259
260 *DLA association with clinical score*
261 The DLA allele or haplotype frequencies did not differ between groups based on
262 clinical score in any of the individual studies (Supplementary Data Table 3), or when all four
263 study groups were combined ($\chi 2$=22.91, df=26, *P*=0.64).
264
265 *DLA association with Leishmania serology*

266 When we compared DLA haplotype frequencies between categories based on
267 serology test results, DLA haplotype frequencies differed between groups only in Study C
268 (Table 3). Specifically, the DLA-DRB1*001:02--DQA1*001:01--DQB1*002:01 haplotype
269 was more common in the high seropositivity group than the low seropositivity group
270 (*P*=0.03, OR=15.4, 95%CI=1.5-170.0) or the medium seropositivity group (*P*=0.025,
271 OR=6.3, 95%CI=1.3-30.5), but frequency of this haplotype did not differ between medium
272 and low seropositivity groups (*P*>0.05). The DLA-DRB1*006:01--DQA1*005:01:1--
273 DQB1*007:01 haplotype was more common in the medium seropositivity group than the
274 high seropositivity group (*P*=0.013). Haplotype frequencies did not differ between groups in
275 studies B or D (*P*>0.05; Supplementary Table 4). Similarly, haplotype frequency between
276 groups did not differ when all groups were combined ($\chi2$=19.03, df=22, *P*=0.65).
277 There was no association with DLA-DRB1 or DLA-DQA1 and seropositivity in any of the
278 groups studied. However, DLA-DQB1 allele 002:01 in study C was more common in the
279 high seropositivity group than the medium seropositivity group (*P*=0.01, odds ratio [OR]
280 =8.0, 95% confidence intervals [CI]=1.6-40.0) or the low seropositivity group (*P*=0.01,
281 OR=15.0, 95% CI=2.02-111.2). Conversely DLA-DQB1 allele 007:01 in study C was more
282 common in the medium group than the high group (*P*=0.01, OR=∞).
283
284 *DLA association with Leishmania DNA concentration in the bone marrow*
285 When we compared DLA haplotype frequencies between categories based on parasite
286 DNA concentration in the bone marrow, the DLA-DRB1*006:01--DQA1*005:01:1--
287 DQB1*007:01 haplotype was more common in the medium group than the low group
288 (*P*=0.02, OR=22.0, 95% CI= 1.5-314.3) only in study B (Table 4), but no other differences
289 existed (Supplementary Table 5). Haplotype frequency between groups did not differ when
290 all studies were combined ($\chi2$=30.16, df =26, *P*=0.26).

291

292 There was no association with DLA-DRB1 or DLA-DQB1 and parasite load in any of
293 the groups studied. However, DLA-DQA1 allele 001:01 was more common in the medium
294 group than the low group in Study C (P=0.01, OR=10.0, 95%CI=1.76-56.9).

295

296 **Discussion**

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

309

310 All dogs in the study were experimentally infected with *L. infantum* by IV injection,
311 which is not necessarily comparable with natural infection by biting sandflies. Additionally,
312 the infective dose of either promastigotes or amastigotes (1×10^6 parasites and 2×10^8
313 parasites, respectively) was much greater than in natural infection, which researchers believe
314 to be approximately 100-1000 parasites per bite (Saridomichelakis, 2009; Rogers, 2012). In
315 IV infection models, researchers believe that parasite dissemination to the organs and

316 therefore clinical signs occur more quickly (Moreno and Alvar, 2002), which means that
317 studies are less time consuming, although not truly representative of natural infection.
318 However, since the dogs are kept in a controlled environment and are exposed to a fixed dose
319 of parasite at a known time point, there are less random effects that could influence the
320 outcome of infection, which is crucial in providing efficacy data for vaccine licencing and
321 potentially useful in evaluating potential genetic factors associated with disease.
322
323 Clinical scores were used to evaluate the nature and severity of clinical signs at
324 different time points following IV infection with *L. infantum*. Most dogs developed some
325 degree of illness during the study and some dogs started to develop clinical signs at 2-3
326 months post-infection, although more severe clinical signs were not evident until around 7
327 months post-infection. Our findings mirror those reported in other studies using IV infection
328 methods, where clinical signs often appear from around 2-4 months post-infection (Carrera et
329 al., 1996; Poot et al., 2005) and clinical outcomes of infection vary between dogs (Nieto et
330 al., 1999; Campino et al., 2000).
331
332 We used clinical scores to quantify the clinical signs observed in dogs over time and
333 to distinguish very sick dogs from those with mild clinical signs. However, dogs with
334 moderate clinical scores were more difficult to evaluate, particularly in the absence of
335 clinicopathological data. Such data could have enabled us to use a clinical staging system
336 such as the Leishvet guidelines or those proposed by the Canine *Leishmania*sis Working
337 Group (Solano-Gallego et al., 2009; Roura et al., 2013), which stage dogs based on clinical
338 signs, clinicopathological abnormalities and *Leishmania* diagnostic testing information.
339 However, clinical staging might not distinguish dogs predisposed to developing clinical
340 disease from those that are more resistant to infection, when dogs are exposed to a high dose

341 of pathogen in experimental infection and thus might not have been useful in phenotyping
342 dogs.
343
344 High *Leishmania* antibody reactivity accompanied by clinical and clinicopathological
345 abnormalities is considered diagnostic for canine leishmaniosis (Solano-Gallego et al., 2011).
346 *Leishmania*-specific antibody levels tended to increase over time regardless of the challenge
347 type. Previous studies have demonstrated that seroconversion following infection is variable
348 with regards to the number of dogs that seroconvert and the time of seroconversion post-
349 infection with promastigote challenge (Nieto et al., 1999; Campino et al., 2000; Paranhos-
350 Silva et al., 2003). Assessment of cell mediated immunity was not performed as the
351 relationship between T-helper phenotypes and disease outcome is still unclear (Maia and
352 Campino, 2012) and thus could have further confounded efforts to categorise dogs based on
353 outcome of infection.
354
355 Quantitative PCR for detection of *Leishmania* DNA was used to confirm the presence
356 of active infection and bone marrow aspirates have greater sensitivity for detection of parasite
357 DNA by qPCR compared with peripheral blood samples (Francino et al., 2006; Hernandez et
358 al., 2015). Most dogs in our study were qPCR positive in the bone marrow by the end of the
359 study, similar to previously published studies (Leandro et al., 2001; Fernandez-Cotrina et al.,
360 2013).
361
362 Dogs in our study displayed variability for all measured parameters, even those dogs
363 that had received the same type of challenge, dose and were assessed at the same time after
364 infection. This might reflect differences in individual dogs' immune responses to *L. infantum*
365 and the ability to control parasite numbers and prevent disease. In this study, the clinical

366 score did not appear to correlate with serology test results or with parasite DNA detection in
367 the bone marrow and, unexpectedly and counter-intuitively, in one study the clinical score
368 was inversely correlated with parasite load in the bone marrow. We did not anticipate this
369 result, as several studies have indicated that dogs with demonstrable clinical signs following
370 natural infection had elevated parasite loads in various tissues, as well as high antibody
371 reactivity (Reis et al., 2006; Dos-Santos et al., 2008; Solano-Gallego et al., 2016). Similarly,
372 *Leishmania*-specific antibody positivity have been shown to correlate with parasite loads in
373 the tissues of dogs naturally infected with *L. infantum* (Reis et al., 2006; Manna et al., 2009;
374 Solano-Gallego et al., 2016). It is possible that the experimental model using Beagle dogs
375 resulted in a more homogenous clinical picture than one might expect with natural infection
376 in a more outbred population. Furthermore, it is possible that some dogs would have
377 developed more severe clinical signs or multi-systemic clinical signs if they had been
378 followed for a longer period of time. It is also possible that clinical score does not adequately
379 separate dogs that are somewhere in the middle of the disease spectrum and that this could be
380 having an impact on lack of correlation between parameters.
381
382 The individual study designs were somewhat variable, which meant that phenotyping
383 dogs for a genetic association study was challenging and groups could not be easily
384 combined. Additionally, as clinical disease was not consistently associated with anti-
385 *Leishmania* antibodies or with parasite load in the bone marrow, we could not categorise
386 dogs, based on both clinical disease, immunological parameters and infection status, so we
387 analysed the relationships of these parameters independently. Alternative phenotyping
388 methods could have assessed *Leishmania*-specific antibodies or *Leishmania* DNA
389 concentration as a quantitative trait, or used all diagnostic parameters in a mixed model.
390 However, as the samples and diagnostic data were obtained from several different studies,

391 there was variability in the time points when these parameters were measured, which would
392 have been difficult to model. The phenotyping method used, while far from perfect, aimed to
393 assess disease expression of each dog compared against the median disease expression of the
394 group at each time point. This method could then be used to distinguish individual dogs with
395 disease expression consistently higher or lower than the group median and could therefore be
396 considered to be highly susceptible or less susceptible for each parameter measured. All
397 genetic association studies were likely to be underpowered as a result of the small sample
398 size in each group and should be repeated in a larger cohort.
399
400 Ten DLA haplotypes were identified in at least three Beagles. While this degree of
401 restriction at the DLA locus is not as marked compared with some breeds, such as the
402 Dobermann and the Rottweiler (Kennedy et al., 2002), it could affect the repertoire of peptide
403 epitopes that can be presented to CD4+ T lymphocytes upon antigenic stimulation. This
404 limited diversity could also influence the response to experimental infection with *L. infantum*
405 and vaccine responses such that a product deemed safe and efficacious in Beagles would not
406 necessarily perform similarly in other dog breeds. Furthermore, there was substantial
407 variation in DLA profile between the different study groups, which indicates that
408 immunogenetic profile has not been a factor in selection of dogs for these vaccine studies.
409 However, differences in DLA profile between studies and within a study could result in
410 variability in response to infection and vaccination between groups that is undesirable when
411 trying to determine the efficacy of a new vaccine.
412
413 None of the study groups differed in DLA haplotype frequencies when assessed
414 against clinical outcomes. One group showed a difference in haplotype frequencies between
415 different serological responses. Specifically, the DLA-DRB1*001:02--DQA1*001:01--

416 DQB1*002:01 haplotype was associated with a sustained elevation of anti-*Leishmania*
417 antibodies over the study period, compared with dogs that had low or fluctuating antibody
418 reactivity over the same period. A previous study examining DLA haplotypes in crossbreed
419 dogs in a *Leishmania* endemic region naturally infected with *Leishmania* reported that the
420 DRB1 genotype 015:02 was associated with increased anti-*Leishmania* antibody levels and
421 PCR positivity in the bone marrow, but did not demonstrate an association with clinical score
422 (Quinnell et al., 2003). This particular DRB1 allele was not present in the Beagle population
423 and therefore could not be assessed. Similarly, the DRB1*001:02 allele found to differ
424 between groups in our study was not one of the common alleles described in a study of
425 natural infection of Brazilian dogs. Interestingly, the haplotype DLA-DRB1*015:01--
426 DQA1*009:01--DQB1*001:01, was found in dogs with high sustained parasite load in the
427 bone marrow, but did not differ in frequency from those showing low parasite loads
428 (*P*=0.06). It is worth noting that DLA-DRB1*015:01 differs by a single amino acid from
429 015:02 as a result of a single base pair substitution (Kennedy et al., 1998).
430
431 Both our study and a previously published study examining naturally infected dogs
432 (Quinnell et al., 2003) found a DLA association with the antibody response, but not with
433 clinical disease expression. DLA genes might influence antigen presentation and impact on
434 the subsequent antibody response to *L. infantum*, but it is possible that there are other host
435 and environmental factors that determine disease expression. It is also possible that the
436 clinical scoring system used is not a sufficiently robust method for distinguishing highly sick
437 individual dogs from those with less severe clinical signs.
438
439 In one group, dogs of another haplotype (DLA-DRB1*006:01--DQA1*005:01:1--
440 DQB1*007:01) that demonstrated intermediate anti-*Leishmania* seropositivity occurred more

441 frequently than in those with high seropositivity. However, in another group, the DLA-
442 DRB1*006:01--DQA1*005:01:1--DQB1*007:01 haplotype occurred more frequently in dogs
443 with an intermediate parasite DNA concentration than in those showing low parasite DNA
444 concentration in the bone marrow over the study period. It is possible that the effect of
445 homozygosity for DLA-DRB1*006:01--DQA1*005:01:1--DQB1*007:01 could be creating a
446 spurious result for this particular haplotype. Therefore, we repeated this analysis using
447 number of dogs rather than number of haplotypes (data not shown) and there was not a
448 significant association with seropositivity, or with parasite load (P >0.05; Supplementary
449 Tables 6 and 7).
450
451 Further work is required in a larger group of dogs receiving a similar experimental
452 challenge of *L. infantum* or in a naturally infected cohort to identify if a true association
453 between DLA genes and outcome of infection with *L. infantum* exists in dogs. Examination
454 of other immune response genes is also warranted and would expand existing knowledge on
455 disease pathogenesis following infection and potentially identify new vaccine targets. The
456 selection of dogs for vaccine studies based on their genetic susceptibility profile might result
457 in less individual variability and more robust trials. Furthermore, early identification of dogs
458 more predisposed to developing clinical disease would facilitate targeted prevention and
459 vaccination strategies in endemic regions.
460

461 **Conclusions**

462 Beagle dogs demonstrated variability in their clinical and serological response to
463 experimental infection with *L. infantum* and in their ability to control parasite load in the
464 tissues, despite some degree of restriction in DLA diversity. No DLA association with
465 clinical score was observed. Investigation of the effect of DLA haplotypes on the outcome of

466 experimental infection revealed a potential haplotype (DRB1*001:02--DQA1*001:01--
467 DQB1*002:01) which was associated with strong serological responses, which are normally
468 counterproductive in this type of intracellular infection, although such an association could
469 not be demonstrated in all groups. Immunogenetic investigation of larger cohorts of dogs,
470 following a standardised experimental challenge is warranted. Immunogenetic analysis could
471 inform vaccine study design and appropriate selection of dogs for such trials, so that vaccine
472 efficacy studies can be more economical and ethical, with a reduction in the number of dogs
473 used.
474

475 **Conflict of interest statement**

476 This research was sponsored by Zoetis, which supplied the samples for genetic
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482

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488

489 **Appendix: Supplementary data**

490

491 Supplementary data associated with this article can be found, in the online version, at
492 doi: ...'
493
494
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496
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605

606

607 **Table 1.**
608 Number of dogs categorised by their disease phenotype according to phenotyping method
609

Phenotyping method	Disease phenotype	Number of dogs (% of group)			
		Study A (*n*=30)	Study B (*n*=17)	Study C (*n*=23)	Study D (*n*=20)
Clinical score	High	17 (57%)	6 (35%)	8 (35%)	6 (30%)
	Medium	8 (27%)	7 (41%)	11 (48%)	10 (50%)
	Low	5 (16%)	4 (24%)	4 (17%)	4 (20%)
Serology	High	[a]	5 (29.5%)	6 (26%)	2 (10%)
	Medium	[a]	7 (41%)	11 (48%)	13 (65%)
	Low	[a]	5 (29.5%)	6 (26%)	5 (25%)
Parasite load(BM)	High	19 (63%)	8 (47%)	5 (22%)	5 (25%)
	Medium	9 (30%)	3 (18%)	12 (52%)	11 (55%)
	Low	2 (7%)	6 (35%)	6 (26%)	4 (20%)

610 [a] Serology not performed in this study

611

612 **Table 2.**
613 DLA haplotypes identified in Beagles experimentally infected with *L. infantum*.
614

Haplotype			Experimental infection study (number of haplotypes)			
DRB1	DQA1	DQB1	Study A	Study B	Study C	Study D
001:01	001:01	002:01	19 [a]	2	5	3
001:02	001:01	002:01	6	0	12 [a]	8
002:01	009:01	001:01	1	2	0	1
006:01	001:01	008:02	0	9 [a]	5	6
006:01	001:01	008:01:1	0	2	0	0
006:01	005:01:1	007:01	15	10	12 [a]	17 [b]
008:01	003:01	004:01	0	6 [a]	3	1
014:01	001:01	008:01:1	0	2	2	0
015:01	009:01	001:01	17 [a]	0	2	2
019:01	004:01	013:03 /017:01	0	0	5	1
Other single haplotypes			2	1	0	1
Total (haplotypes)			60	34	46	40
Total number of dogs			30	17	23	20
Total number of homozygous dogs			2	3	2	3

615 [a] One homozygous dog with this haplotype
616 [b] Three homozygous dogs with this haplotype

617 **Table 3.**

618 DLA haplotypes in Study C phenotyped according to serology test results [a]

Haplotype			Number of haplotypes			
DRB1	DQA1	DQB1	High	Medium	Low	*P*
001:01	001:01	002:01	2	2	1	0.7
001:02	001:01	002:01	7[b]	4	1	0.01
006:01	001:01	008:02	1	1	3	0.18
006:01	005:01:1	007:01	0	9[b]	3	0.03
008:01	003:01	004:01	0	1	2	0.23
014:01	001:01	008:01:1	0	2	0	0.49
015:01	009:01	001:01	0	1	1	0.99
019:01	004:01	013:03/0 17:01	2	2	1	0.7
	Total (haplotypes)		12	22	12	
	Total (dogs)		6	11	6	

619 [a] Dogs with *Leishmania*-specific IgG (ELISA Units) above the median in 70% of the study
620 time points are categorised as high susceptibility, Dogs with *Leishmania*-specific IgG levels
621 below the median in 70% of the study time points are categorised as low susceptibility. Each
622 haplotype is made up of three loci- DLA-DRB1, DLA-DQA1 and DLA-DQB1. *P* values
623 comparing all three groups were determined by the Fisher's exact probability test.
624 [b] One dog homozygous for this haplotype
625 [c] $P<0.05$

626 **Table 4.**
627 DLA haplotypes in Study B phenotyped according to *Leishmania* DNA concentration in the
628 bone marrow [a]
629

Haplotype			Number of haplotypes			P
DRB1	DQA1	DQB1	High	Medium	Low	
001:01	001:01	002:01	1	0	1	0.99
002:01	009:01	001:01	1	0	1	0.99
006:01	001:01	008:02	3	1	5[b]	0.45
006:01	001:01	008:011	0	1	1	0.27
006:01	005:01:1	007:01	5	4[b]	1	0.03[c]
008:01	003:01	004:01	4[b]	0	2	0.52
014:01	001:01	008:01:1	1	0	1	0.99
Other single haplotypes			1	0	0	N/A
Total (haplotypes)			16	6	12	
Total (dogs)			8	3	6	

630 N/A, Not applicable
631 [a] Dogs with *Leishmania* DNA concentration (genome copy/mL) in the bone marrow above
632 the median in 70% of the study time points are categorised as high susceptibility. Dogs with
633 *Leishmani*a DNA concentration in the bone marrow below the median in 70% of the study
634 time points are categorised as low susceptibility. Each haplotype is made up of three loci-
635 DLA-DRB1, DLA_DQA1 and DLA-DQB1. P values comparing all three groups were
636 determined by the Fisher's exact probability test.
637 [b] One dog homozygous for this haplotype
638 [c] $P<0.05$
639

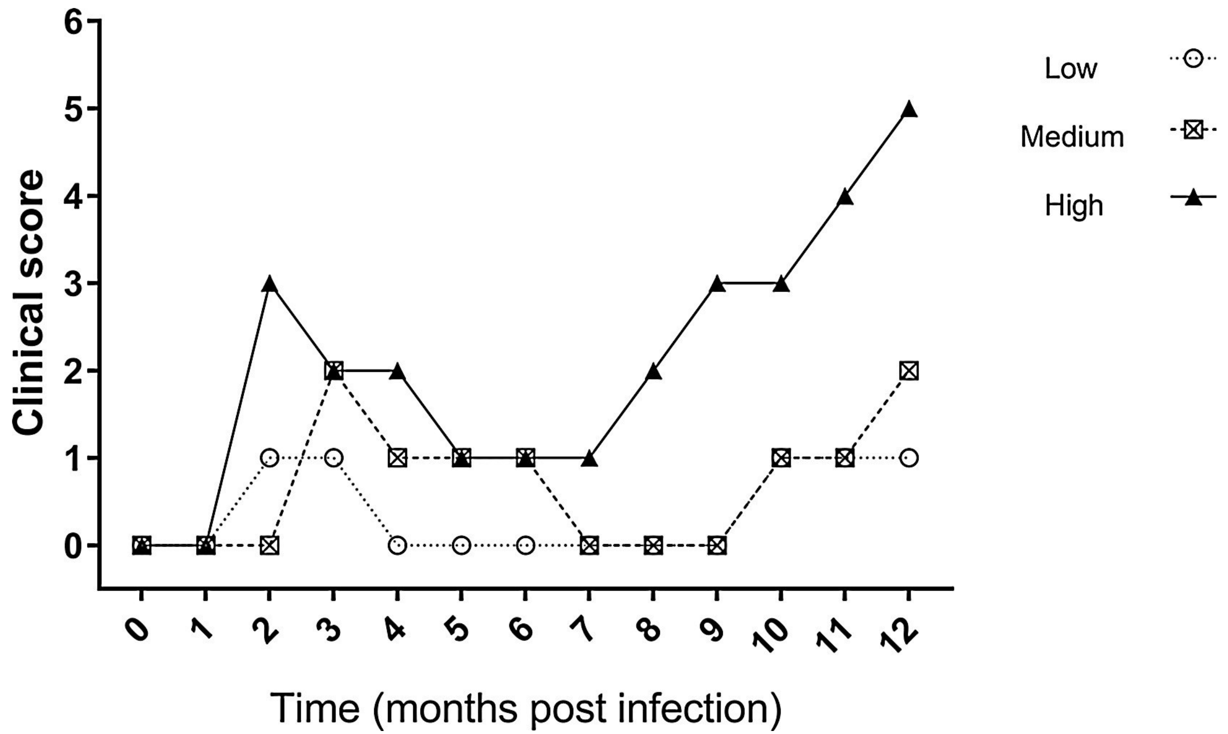
640 **Figure legends**

641

642 Fig. 1. Clinical score over time for representative dogs in study B. Dogs in the high category

643 had scores above the median in >70% of the time points. Dogs in the low category had scores

644 below the median in >70% of the time points.



645

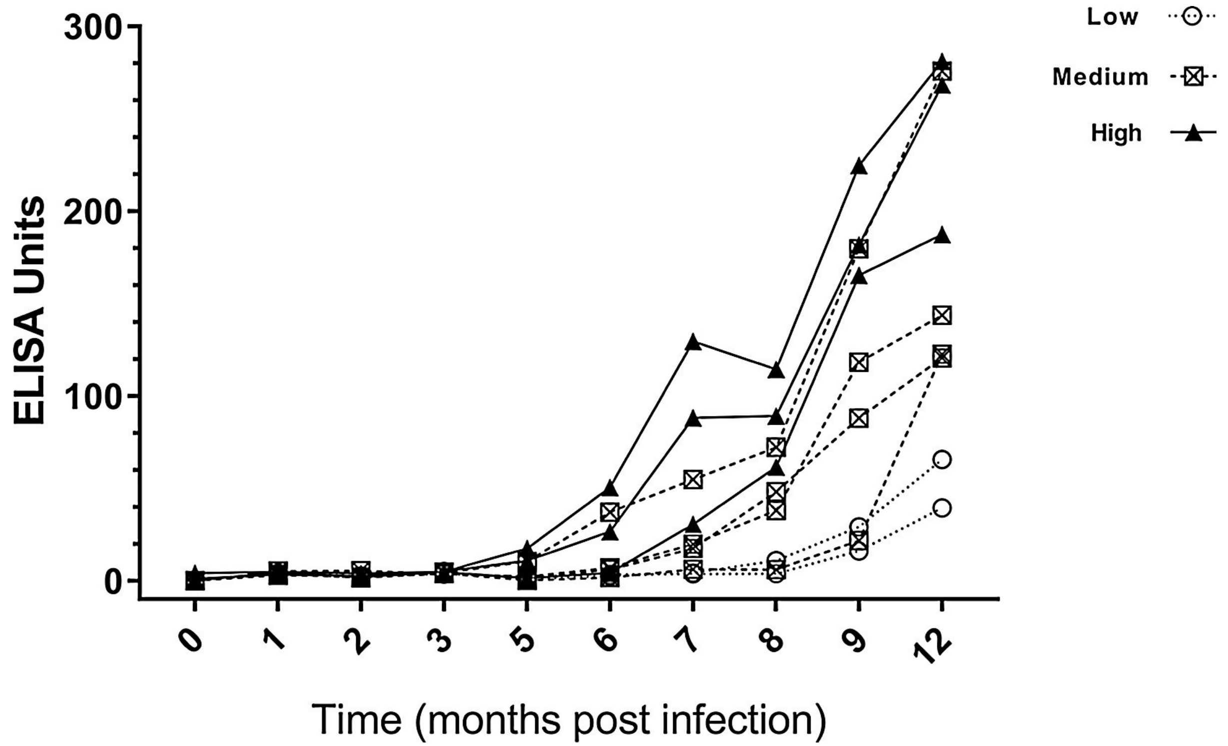
646

647 Fig. 2. Serology results for representative dogs in study B. *Leishmania*-specific IgG was

648 measured by ELISA. Dogs in the high category had scores above the median in >70% of the

649 time points. Dogs in the low category had scores below the median in >70% of the time

650 points.



651

652

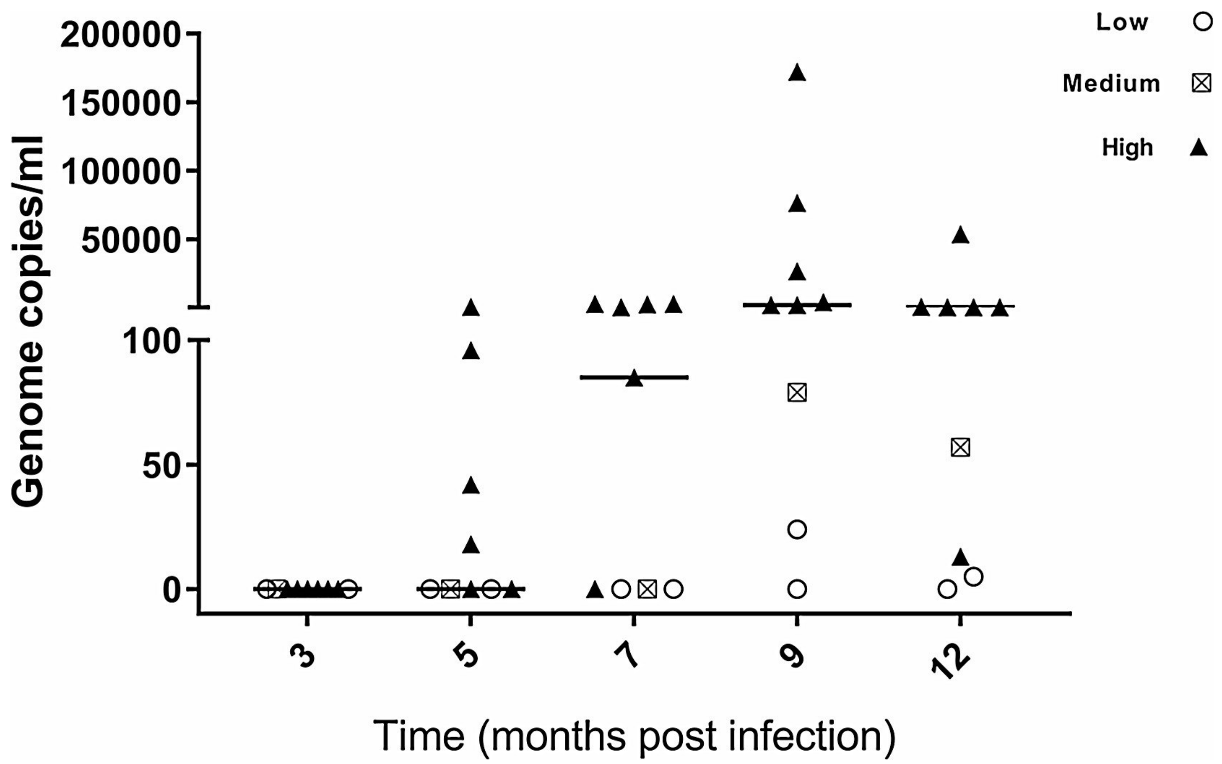
653 Fig. 3. Parasite load in the bone marrow for representative dogs in study B. *Leishmania* DNA

654 in the bone marrow was measured by qPCR. The median score for each time point is

655 represented by a horizontal line. Dogs in the high category had scores above the median in

656 >70% of the time points. Dogs in the low category had scores below the median in >70% of

657 the time points.



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