TITLE: An investigation of polymorphisms in innate and adaptive immune response genes in canine leishmaniosis

AUTHORS: F. Soutter, L. Solano-Gallego, C. Attipa, L. Gradoni, E. Fiorentino, V. Foglia Manzillo, G. Oliva, S. Tasker, C. Helps, B. Catchpole

JOURNAL: Veterinary Parasitology

PUBLISHER: Elsevier

PUBLICATION DATE: 26 April 2019 (online)

DOI: https://doi.org/10.1016/j.vetpar.2019.04.011

Original Article

An investigation of polymorphisms in innate and adaptive immune response genes in canine leishmaniosis

Francesca Soutter [a,*], Laia Solano-Gallego [b] Charalampos Attipa[a,c], Luigi Gradoni[d], Eleonora Fiorentino[d], Valentina Foglia Manzillo[e], Gaetano Oliva[e], Séverine Tasker[f], Chris Helps[f], Brian Catchpole [a]

[a] *Department of Pathobiology and Population Sciences, Royal Veterinary College, North Mymms, Hertfordshire, AL9 7TA, UK*

[b] *Departament de Medicina i Cirurgia Animal, Facultat de Veterinària, Universitat Autònoma de Barcelona, Barcelona, Spain*

[c] *Cyvets Veterinary Center, Paphos, Cyprus.*

[d] *Unit of Vector-Borne Diseases, Department of Infectious Diseases, Istituto Superiore di Sanità, Rome, Italy*

[e] *Dipartimento di Medicina Veterinaria e Produzioni Animali, Naples University, Naples, Italy*

[f] *Molecular Diagnostic Unit, Diagnostic Laboratories, Langford Vets, University of Bristol, BS40 5DU, UK*

* Corresponding author. Tel.: +44 170 766 9457

E-mail address: fsoutter@rvc.ac.uk (F. Soutter).

Abstract

The outcome of infection with *Leishmania infantum* in dogs is variable, which is thought to be due to the nature of the immune response mounted by the host. As a consequence, the clinical signs and severity of canine leishmaniosis vary between individual dogs. Host immunogenetic factors might play an important role in determining the outcome of infection. The aim of this study was to examine polymorphisms in innate and adaptive immune response genes, to determine whether any of these were associated with susceptibility or resistance to *L. infantum* infection. Genomic DNA was obtained from two groups: pet dogs in endemic regions of Europe and a group of Beagles exposed to sand fly infection as part of a vaccine study. Genotyping was performed using a SNP (single nucleotide polymorphism) array for selected immune response genes. The first part of the study compared 62 clinical cases with 101 clinically unaffected dogs that were seronegative for *Leishmania* antibodies. One SNP in the *CIITA* gene demonstrated a significantly higher minor allele frequency in the case group, compared with the control group at the individual SNP level after permutation, but was not significant after correction for multiple testing. The second part of the study examined 48 Beagle dogs exposed to *L. infantum* over two transmission seasons. Twenty-seven dogs with a resistant phenotype (no evidence of clinical disease, seronegative at the end of the study period, negative on lymph node culture and only transiently PCR positive in bone marrow) were compared with 21 dogs demonstrating a susceptible phenotype (clinical disease, seropositive, positive lymph node culture and consistently PCR positive in bone marrow). Three SNPs in *TLR3*, two SNPs in *PTPN22* and one SNP in *TLR4* and *IL1A* were associated with the susceptible

phenotype in the Beagle group at the individual SNP level after permutation analysis, but were not significant after correction for multiple testing. Further validation of these SNPs is required in a larger cohort of dogs, ideally with extreme phenotypes to confirm an association with the outcome of *L. infantum* infection.

Keywords

***Leishmania*, Genetics, Dogs, Innate, Adaptive**

Abbreviations

CIITA: class II major histocompatibility complex transactivator, C6: complement C6, C7: complement C7, CLEC16A: C-type lectin domain family 16 member A, DEXI: dexamethasone-induced protein, DLA: dog leukocyte antigen, EDTA: ethylenediaminetetraacetic acid, ELISA: enzyme linked immuno-absorbant assay, EU: ELISA units, GAPDH: glyceraldehyde 3-phosphate dehydrogenase, gDNA: genomic DNA, GSPL: glycosphingophospholipids, GWAS: genome wide association study, HWE: Hardy Weinberg equilibrium, IFAT: indirect immunofluorescence assay, IFN: interferon IgG: immunoglobulin G, IL: interleukin, IL1A: interleukin-1 alpha,IL2RA: interleukin-2 receptor alpha, IL7R: interleukin-7 receptor,IL15RA: interleukin-15 receptor alpha, LIFR: leukemia inhibitor factor receptor alpha, LPS: lipopolysaccharide, MAF: minor allele frequency, MHC: major histocompatability complex, NO: nitric oxide, PTPN22: protein tyrosine phosphatase non-receptor type 22, qPCR: quantitative PCR, SD: standard deviation, SLC11A1: solute carrier family 11 (formally NRAMP), SOCS1: suppressor of cytokine signalling 1, SNP: single nucleotide polymorphism, TLR: toll-like receptor

Introduction

Canine leishmaniosis is caused by the protozoan parasite *Leishmania infantum*, which is also responsible for zoonotic visceral and cutaneous leishmaniosis in humans (Gramiccia and Gradoni, 2005). *L. infantum* is endemic in the Mediterranean basin,

Central and South America and parts of Africa and Asia (Palatnik-de-Sousa and Day, 2011), with evidence of emerging disease elsewhere (Maia and Cardoso, 2015), as a result of increasing phlebotomine sand fly vector distribution and dogs travelling to and from endemic areas potentially spreading disease to Northern areas of Europe (Shaw et al., 2009).

There is a spectrum of clinical leishmaniosis in dogs, varying from mild skin lesions and localised lymphadenomegaly to multi-organ involvement and renal failure (Koutinas et al., 1999). Disease progression and severity of clinical signs, and/or clinicopathological abnormalities observed, vary between individual dogs, which suggests that some dogs might be more resistant to disease than others. Diagnosis of canine leishmaniosis is based on the presence of clinical signs and clinicopathological abnormalities compatible with disease, alongside diagnostic methods of determining infection with *L. infantum* (Solano-Gallego et al., 2009). The most commonly used indirect diagnostic methods are serological tests, including the indirect immunofluorescence assay (IFAT) and enzyme linked immunoabsorbant assay (ELISA), which determine the presence of *Leishmania* antibodies in the serum (Paltrinieri et al., 2010). High anti-*Leishmania* antibody reactivity has been associated with high parasite loads and clinical disease (Solano-Gallego et al., 2001). Direct demonstration of the parasite by cytological examination of affected tissues (Paltrinieri et al., 2010) or detection of *Leishmania* DNA in the tissues, using PCR or quantitative PCR (qPCR), are also used (Cortes et al., 2004; Francino et al., 2006). A definitive diagnosis of canine leishmaniosis can be difficult to achieve, although there is a high index of suspicion for individuals with clinical signs of overt leishmaniosis and a highly positive serology result (Paltrinieri et al., 2010). However, when dogs

present with a low clinical suspicion index, or where anti- *Leishmania* antibody reactivity is low, multiple diagnostic tests might be required to confirm the diagnosis (Solano-Gallego et al., 2009; Paltrinieri et al., 2010).

A number of host and parasite factors seem to play a role in determining the outcome of infection. The host immune response might be particularly important in disease outcome, with $CD4^+$ T helper type 1 (Th1) lymphocytes and their ability to induce macrophages to kill intracellular amastigotes via production of IFN-γ considered to be crucial in controlling infection (Pinelli et al., 1994). Although the immune response in dogs affected with leishmaniosis has been studied in some detail, knowledge gaps still remain in terms of the precise mechanisms involved in disease susceptibility/resistance.

It has been suggested that host immunogenetic factors might determine whether the immune response is protective or not. A previous study examined dog leukocyte antigen (DLA) genes, which encode MHC Class II molecules and found an association between one particular DLA haplotype and increased anti-*Leishmania* IgG and presence of *Leishmania* DNA in the bone marrow (Quinnell et al., 2003). Genome wide association studies (GWAS) have also been performed more recently, in which polymorphisms on chromosomes 1 and 4 were found to be significant and a potential locus on chromosome 4 that includes immune response genes (*IL7R, LIFR, C6 a*nd *C7*) (Quilez et al., 2012). Two further SNPs have been associated with leishmaniosis, one located on chromosome 2, proposed to be in linkage with a causal variant in the *IL2RA* or *IL15RA* gene and another on chromosome 1, which might be in linkage with a gene involved in Notch signalling (Utsunomiya et al., 2015). A more recent GWAS identified SNPs on chromosome 20 to be associated with increased TNF-α

126 concentration in *Leishmania* antigen stimulated lymphocytes, whilst SNPs on
127 chromosome 17 were associated with increased IL-10 concentration (Cortes et al.,
128 2012).

129 The aim of this study was to interrogate polymorphisms in candidate innate and
130 adaptive immune response genes in dogs naturally infected with *L. infantum* to
131 determine whether there are associations with clinical disease and/or infection status.

132 **Methods**

133

134 *Canine population and study design*

135

136 The study dogs consisted of two populations that were analysed separately. The first
137 study comprised of pet dogs from two *Leishmania* endemic regions of Europe
138 consisting of clinical cases and controls. The second study comprised of Beagle dogs
139 kept outdoors in an endemic region and thus exposed to sand flies and *Leishmania*
140 infection for two years and regularly monitored (longitudinal study).

141

142 In Study 1, blood samples were obtained from dogs that presented to first-opinion
143 veterinary practices, one in Paphos, Cyprus and the other in Zaragoza, Spain. Dogs
144 vaccinated with Canileish (Virbac) or with a history of immunosuppressive therapy
145 were excluded. Clinical cases of leishmaniosis were identified based on clinical
146 examination and confirmation testing by PCR and serology. Samples from clinically

147 healthy control dogs, breed and age matched where possible, resident in the same
148 endemic regions were also recruited through these veterinary practices. Signed
149 informed consent was obtained from owners for permission to use any excess blood
150 for clinical research after completion of diagnostic testing. Approval was granted from
151 the Clinical Research Ethics Review Board of the Royal Veterinary College (reference
152 number URN 2014 1292; date of approval 03/09/2014) for use of the samples in
153 research.

154 In Study 2, residual genomic DNA samples were provided from Beagles enrolled in a
155 natural infection model, where dogs were studied over a 2 year period. Clinical and
156 clinicopathological abnormalities were observed over the period of the study and
157 diagnostic testing was performed every 3 months after an initial 6 month exposure
158 period. *Leishmania* testing included Immunofluorescence Antibody Test (IFAT) and
159 nested PCR on the bone marrow and lymph node parasite culture as previously
160 described (Oliva et al., 2014). The study was approved by the Veterinary Board of the
161 Italian Ministry of Health following the European Directive 86/609/EEC, adopted by
162 the Italian Government with the Law 116/1992. Approval was granted from the
163 Clinical Research Ethics Review Board of the Royal Veterinary College (approval
164 number URN 2015 1329; date of approval 05/03/2015) for the use of these samples in
165 research.

166

167 *Diagnostic procedures*

168

169 *i. Culture technique*

Parasite isolation by culture was performed on lymph node aspirates from dogs in Study 2. Briefly, lymph node aspirates were cultured in Evans' modified Tobie's medium at 22.5°C and were examined for promastigote growth after 1 month (Oliva et al., 2006).

ii. Molecular analyses

Real-time qPCR for *L. infantum* kinetoplast DNA was performed for Study 1 (Shaw et al., 2009). Genomic DNA (gDNA) was extracted from EDTA blood samples using the GenElute Blood Genomic DNA Kit (Sigma-Aldrich, Dorset, UK) according to the manufacturer's instructions. This was submitted to the Acarus laboratory (Molecular Diagnostic Unit, Langford Vets, Bristol) for qPCR testing. Results were normalised against the median GAPDH reference value for the group. Dogs were categorised as qPCR negative for *Leishmania* kinetoplast DNA if a CT value could not be determined for the sample. Samples were categorised as borderline positive if they had a CT value >35 and considered positive if the CT value ≤35.

Nested PCR for *L. infantum* kinetoplast DNA was performed on gDNA extracted from bone marrow samples from dogs in Study 2 as previously described (Oliva et al., 2006). Bone marrow samples from *Leishmania*-free dogs were used as negative controls in each step of the procedure. The amplification products were analysed on

1.5% (w/v) agarose gels and visualized under UV light. Positive samples yielded a PCR product of 358 bp.

iii. Serological techniques

ELISA testing was performed on serum samples from Study 1 dogs to assess the presence of anti-*Leishmania* antibodies as previously described (Solano-Gallego et al., 2014). Results were quantified as ELISA units (EU), relative to the calibrator (arbitrarily set at 100 EU). The positive cut-off value had previously been established at 35 EU (mean + 4 SD of values from 80 dogs from a non- endemic area). Positive sera were classified as borderline (35-≤37 EU), low (37-≤150 EU) medium (150-≤300 EU) or high (>300 EU).

The IFAT was performed on serum samples from Study 2 dogs. Briefly, *L. infantum* parasites (MHOM/TN/1980/IPT-1) were fixed to microscope slides. Serial dilutions of serum were added to the slides and incubated for 30 min at 35-37°C. Serum antibody reactivity to parasites was detected using a fluorescent secondary rabbit anti-dog IgG antibody (Sigma-Aldrich). The antibody titre represents the final dilution at which at least 50% of the parasites were visible by fluorescence. Titres ≥ 1:160 were considered to be positive for infection (Oliva et al., 2014).

Genotyping of candidate canine immune response genes and data analysis

Sequenom MassARRAY genotyping was performed at the Centre for Integrated Genomic Medical Research, University of Manchester as previously described (Short et al., 2007). Twenty-four candidate genes were selected, consisting of both innate and adaptive immune response genes across different chromosomes (Supplementary Table 1). Sixty-five SNPs had been reported previously (Supplementary Table 2) and a further 47 SNPs had been identified by sequence-based typing for other genetic studies undertaken at the Royal Veterinary College (Supplementary Table 3).

The data was analysed using PLINK whole genome data analysis toolset version 1.07 (http://pngu.mgh.harvard.edu/~purcell/plink/) (Purcell et al., 2007). Results were filtered according to the following criteria for quality control purposes: SNPs with a minor allele frequency (MAF) below 5% and a call rate below 90% were excluded from the analysis. Individuals with more than 10% of the SNP information missing (low genotyping rate) were also excluded from the study. Hardy Weinberg equilibrium (HWE) was assessed for each SNP. Whilst HWE amongst the case population can be indicative of selection, deviation from HWE in the controls can be a result of poor genotyping of these SNPs and HWE was therefore assessed in the Study 1 control population.

Information about the chromosomal location of each SNP included in the array was included in the map document provided with the analysis results. This was based on the NCBI dog genome assembly, version 3.1 (http://www.ncbi.nlm.nih.gov/genome/85).

SNPs were tested for association using Chi square analysis or Fisher's exact test. SNPs were considered as candidate for further investigation if the p value was below the significance cut-off $p<0.05$. Corrected p values for multiple testing were obtained after 1000 permutations. For each permutation the maximum statistic across all SNPs was recorded and from this distribution of maximum statistics, the statistic in the top 5% is used to give the corrected p value. Linkage disequilibrium and haplotype assignment was performed in Haploview 4.2 (Barrett et al., 2005). Haplotypes were tested for association using logistic regression in PLINK.

Results

Diagnostic testing and case definition

The first study examined a heterogenous group of dogs that presented to first opinion practices in two geographically distinct regions where *L. infantum* is endemic. Sixty-two cases of leishmaniosis were recruited (50 dogs from Cyprus and 12 dogs from Spain). One hundred and one controls were recruited (90 dogs from Cyprus and 11 dogs from Spain). The clinical signs observed in the cases were variable (Supplementary Figure 1). The most common clinical abnormalities observed were lymphadenomegaly (enlargement of the peripheral lymph nodes), weight loss and skin lesions. Qualitative assessment of clinicopathological abnormalities as assessed by veterinarians from endemic regions based on in-house biochemistry, complete blood

count and urinalysis was available (Supplementary figure 2) and anaemia and hyperproteinaemia, hyperglobulinaemia and hypoalbuminaemia were the most commonly described abnormalities.

Serological testing revealed that all leishmaniosis cases (n=62) were highly positive (>350 EU) using the ELISA. Fifty-five of the 62 dogs were qPCR positive for *Leishmania* kinetoplast DNA in the blood, 4 dogs were borderline positive and 3 dogs were qPCR negative (Figure 1). The majority of dogs (85/101) in the control group were negative in both ELISA and qPCR tests. Two control dogs were borderline positive for *Leishmania* antibodies by ELISA but qPCR negative and serum was not available for testing in 4 dogs, but these were qPCR negative. Ten dogs were positive by qPCR (7 of which were borderline) but were all ELISA negative.

In Study 2, clinical and diagnostic test information was provided for 48 Beagle dogs selected from a larger research study designed to investigate susceptibility to *L. infantum* infection over a two-year period. Twenty-seven dogs were considered to have a resistant phenotype as they did not display any clinical or clinicopathological abnormalities for the duration of the study, were only transiently *Leishmania* DNA positive in the bone marrow, were negative on lymph node culture and IFAT negative at the end of the study period. In contrast, 21 dogs were considered to have a susceptible phenotype as they demonstrated clinical and clinicopathological abnormalities compatible with leishmaniosis, first detected 6 to 20 months from commencement of the study, remained consistently *Leishmania* DNA positive in the bone marrow, were positive on lymph node culture and were IFAT positive at the end of the study period.

279

280 *SNP array analysis*

281

282 Seventy-three SNPs were included in the final analysis after exclusions for low MAF
283 (<5%), call rate below 90% or lack of variability. Four SNPs significantly deviated
284 from HWE (P<0.00001) in the control population; *TLR1* c.1665T>C, *TLR1*
285 c.1776T>C, *IL6* c.572A>G and *IL10* c.-1330G>A.

286 Linkage disequilibrium between SNPs was estimated using D', a normalised
287 measure of allele association and by r2, the correlation coefficient between 2 SNPs.
288 Multiple SNPs appeared to be in linkage disequilibrium and haplotype blocks were
289 assigned in Haploview based on D' confidence intervals as described previously
290 (Gabriel et al., 2002).

291

292 *Case-control association study: Study 1*

293

294 After initial analysis, 6 individuals were excluded due to a genotyping rate of less than
295 90% (2 cases, 4 controls). The genotyping rate in the remaining individuals was
296 95.2%. The final analysis was therefore performed on 60 clinical cases and 97
297 controls. Two SNPs showed significantly higher MAFs in the case group compared
298 with the control group; *CIITA* c.2595C>T (p=0.008) and *IL6* c.572A>G (p=0.008)
299 (Table 2). The SNP *IL6* c.572A>G was not in HWE and no dogs were found to be
300 heterozygous at that position in our study population. After permutation was

performed, only one SNP was significant at the individual SNP level, *CIITA* c.2595C>T (p=0.036) and neither SNP was significant after the correction for multiple testing, implemented during permutation (p>0.05).

There were no significant differences in genotype frequencies between case and control group for any of the SNPs and no evidence of a significant dominant or recessive penetrance model for any of the SNPs (significance level p<0.05). The *CIITA* c.2595C>T SNP did not demonstrate a significant difference in genotypes between cases and controls (p=0.080). A recessive model for this SNP appeared to be the best fit, but there was no significant difference in frequency of the TT genotype or in the combined frequency of CT and CC genotypes between cases and controls (p=0.053).

Case-control association study: Study 2

Three individuals were excluded from the genetic analysis, due to having a genotyping rate <90% (1 susceptible, 2 resistant phenotypes). Four SNPs showed significantly different MAFs in the susceptible phenotype group compared with the resistant phenotype group (Table 2). Two SNPs had significantly higher MAFs in the susceptible group; *TLR3* c.369C>T (p=0.020) and *TLR4* c.1795G>A (p=0.036). In contrast, two SNPs had significantly higher MAFs in the resistant group; *TLR3* c.1380T>C (p=0.015) and *TLR3* c.1104T>C (p=0.015). After permutation, all four SNPs were still significant at the individual SNP level (p<0.01) and three more SNPs were significant comparing the two groups; *PTPN22* c.88-39G>A (p=0.040), *PTPN22*

c.915+87T>C (p=0.047) and *IL1A* c.-151A>C (p=0.048). However, after correction for multiple testing implemented during permutation, there was no significant difference seen in any of these SNPs comparing the two groups (p>0.05).

The SNPs in *TLR3* and *TLR4* that demonstrated significantly different allele frequencies were also significant when genotype frequencies were assessed between the two groups (Table 3); *TLR3* c.369C>T (p=0.015), *TLR3* c.1104T>C (p=0.011), *TLR3* c.1380T>C (p=0.011) and *TLR4* c.1795G>A (p=0.033). Two SNPs in *PTPN22* also demonstrated significant differences in genotype frequency between groups; *PTPN22* c.-515T>C (p=0.016) and *PTPN22* c.88-39G>A (p=0.035). After permutation all five SNPs were still significant at the individual SNP level (p<0.05). However, after the correction for multiple testing implemented during permutation there was no significant difference between groups for any of these SNPs (p>0.05).

The *TLR3* SNPs, *TLR3* c.1380T>C and *TLR3* c.1104T>C were in linkage disequilibrium (D'=1) and formed a haplotype. There was a significant difference in the frequency of the *TLR3* CC haplotype in the resistant phenotype dogs compared with the susceptible phenotype dogs, with the CC haplotype showing decreased odds of disease (OR= 0.207, p=0.010) (Table 4). Haplotypes were still significant at the individual haplotype level after permutation (p=0.005). However, after correction for multiple testing implemented during permutation, there was no significant difference seen between groups (p=0.092).

The two *PTPN22* SNPs, *PTPN22* c.88-39G>A and *PTPN22* c.915+87T>C were also in linkage disequilibrium (D'=1) and formed a haplotype with one other SNP, *PTPN22* c.-515T>C, which did not appear to be associated at the individual SNP

level. The TAT haplotype showed decreased odds of disease (OR= 0.231, p=0.033), whilst the CGT and CGC haplotypes were not significantly associated with disease (p>0.05). The TAT haplotype was significant at the individual haplotype level after permutation was implemented (p=0.039) but not after correction for multiple testing (p=0.326).

Discussion

Polymorphisms in innate and adaptive immune response genes were examined in different dog populations exposed to *L. infantum* infection to determine whether any of these were associated with disease susceptibility. Although some SNPs showed a significant association with the disease phenotype, these did not reach statistical significance after correction for multiple testing.

A case-control study, performed using samples from a heterogeneous population of client-owned dogs, revealed a SNP (c.2595C>T) in the *CIITA* gene to be associated with canine leishmaniosis. *CIITA* is a key transcriptional activator of MHC Class II, with studies in *CIITA* knockout mice demonstrating significantly lower MHC Class II expression in lymphoid tissues compared with wild type mice (Itoh-Lindstrom et al., 1999). There is evidence of *CIITA* gene variation influencing susceptibility to other infectious diseases in humans, with promoter polymorphisms being associated with persistent infection with hepatitis B virus (Zhang et al., 2007). Furthermore, a recent GWAS identified *CIITA* as a susceptibility gene for leprosy, which, like *L. infantum,* is an intracellular pathogen (Liu et al., 2015).

The *CIITA* c.2595C>T SNP could be in linkage disequilibrium with an as yet unidentified polymorphism in the *CIITA* gene or another gene located nearby on chromosome 6. Other genes, on the same chromosome, which might contain causal variants include *CLEC16A*, encoding a membrane associated endosomal protein, *DEXI*, which encodes a protein of unknown function and *SOCS1*, a suppressor of cytokine signalling; all of which have been found to be associated with immune-mediated disease in humans (Davison et al., 2012). Future studies should interrogate multiple SNPs in this region, to understand which genes, if any, might be of importance in susceptibility to canine leishmaniosis.

Three SNPs in *TLR3* were found to be associated with the disease phenotype in Beagle dogs; two of which were in linkage disequilibrium and did not appear to have independent effects. A significant association with disease was also observed for a SNP in *TLR4*. Although TLR3 recognises double stranded RNA, and is thus important for recognition of viral pathogens, there is some evidence that TLR3 might also recognise *Leishmania* parasites. One study indicated that by inhibiting expression of *TLR3* by RNA interference production of nitric oxide (NO) and TNF-α by macrophages infected *in vitro* with *L. donovani* promastigotes was reduced (Flandin et al., 2006). Furthermore, a recent study revealed a positive correlation between *TLR3* expression and parasite density in the skin of dogs in early experimental infection with *L. infantum* (Hosein et al., 2015).

Potential *Leishmania* ligands for TLR4 are glycosphingophospholipids (GSPL), which have been shown to induce a TLR4 mediated inflammatory response and parasite clearance of *L.donovani* in mice (Karmakar et al., 2012). In mouse models, TLR4 is key to controlling the number of *L.major* parasites (Kropf et al., 2004a;

2004b). In dogs infected with *L. infantum*, the role of TLR4 is unclear; a recent study demonstrated *TLR4* expression in the lymph node and spleen was reduced in infected dogs, compared with uninfected controls (Hosein et al., 2015).

Two SNPs in the *PTPN22* gene appeared to be associated with disease at the individual SNP level, and one other *PTPN22* SNP was significant at the genotype level. *PTPN22* is a susceptibility gene for immune-mediated diseases in humans (Criswell et al., 2005) and with Type 1 diabetes and hypoadrenocorticism in the dog (Short et al., 2007; 2013). PTPN22 is believed to inhibit activation of T cells by dephosphorylation of signal transduction mediators (Stanford and Bottini, 2014), however, the role of PTPN22 has not been investigated with respect to leishmaniosis.

The SNPs associated with disease susceptibility, and the genes in which they were located, were different between the two studies. These differences could be due to the breed profiles and nature of the two studies. Furthermore, in Study 1, the control group of dogs were mostly negative by both ELISA and qPCR testing. These dogs are assumed to have been exposed to *L. infantum* infected sand flies, since they lived in endemic regions, however exposure to sand flies was likely variable due to differences in owner lifestyle. Test sensitivity for *Leishmania* DNA is thought to be low in peripheral blood, when compared with other tissues (Maia and Campino, 2008) and it is therefore possible that these dogs were infected at a low level that could not be detected. An ELISA for the detection of IgG antibodies against sand fly saliva antigens has been shown to correlate with the number of feeding events (Hostomska et al., 2008; Vlkova et al., 2011) and could have been used to confirm exposure to sand flies if not exposure to *L. infantum*. A small number of dogs within the control group were positive by either ELISA or by qPCR, but did not display any clinical signs and

were possibly more representative of resistant dogs provided they remained asymptomatic. Disease progression for these infected but clinically healthy dogs is variable, with longitudinal studies suggesting that some dogs develop severe disease in the short to medium term whereas other dogs remain free from clinical signs for long periods or even indefinitely (Quinnell et al., 2001; Oliva et al., 2006). There are other limitations to this genetic study in terms of the sample size and potential population stratification, which were difficult to overcome in terms of the availability of suitable samples from dogs in endemic regions. Use of a larger number of control dogs might have increased the power and reduced stratification effects (Cardon and Bell, 2001). The Beagle dogs were selected from a larger trial population and were considered to represent extreme phenotypes in terms of resistance and susceptibility to *Leishmania* infection.

Conclusions

Although the study was likely to be underpowered, as a result of small sample size, several genes of interest have been identified that could be involved in susceptibility to canine leishmaniosis. Identification of immune response genes involved in disease susceptibility could inform breeding and disease prevention strategies in the future, as well as more targeted selection of dogs for vaccine challenge studies. Furthermore, these susceptibility genes might represent good targets for manipulation (e.g. via use of specific adjuvants) in development of immunomodulatory therapies and vaccines.

Declaration of interest

Ethics approval and consent to participate

Samples from Study 1 were residual samples taken under the Veterinary Surgeons Act (1966). Signed informed consent was obtained from owners for permission to use any excess blood for clinical research after completion of diagnostic testing. Approval was granted from the Royal Veterinary College Ethics Committee, reference number URN 2014 1292 for sampling the dogs and for use of the samples in research.

Samples from Study 2 were residual samples provided from studies previously undertaken and approved by the Veterinary Board of the Italian Ministry of Health following the European Directive 86/609/EEC, adopted by the Italian Government with the Law 116/1992. Approval was granted from the Royal Veterinary College Ethics Committee, reference number URN 2015 1329 for the use of the samples in research.

Competing interests

ST and CH work for the Diagnostic Laboratories, Langford Vets, University of Bristol. The Laboratories provide a range of commercial diagnostic services including ELISA and qPCR testing for canine leishmaniosis.

Funding

This study was supported by a Biotechnology and Biological Sciences Research Council (BBSRC) Collaborative Awards in Science and Engineering (CASE) studentship (BB/I015655/1) in partnership with Zoetis.

Authors contributions

FS, LSG and BC were involved in study conception and design and co-ordinated the experiments. LSG and FS designed a collection protocol and CA collected samples for Group 1. LG designed a collection protocol, collected samples and performed cultures for Group 2; EF extracted DNA and performed PCR and IFAT for this group. VFM and GO collected and evaluated clinical and clinicopathological parameters from Group 2. FS extracted the DNA and performed ELISA analysis for study 1 and performed the genetic and statistical analysis for both studies. CH and ST co-ordinated the qPCR work for study 1. FS and BC wrote the manuscript with input from all the authors. All authors read and approved the final manuscript.

Acknowledgements

The authors would like to thank the veterinarians in Cyprus and Spain who helped with sample collection. They would also like to thank Sergio Villanueva Saz who helped facilitate sample collection in Spain and Dave Morris who ran the Leishmania qPCR for study 1. Thanks also to Lorna Kennedy, Bill Ollier, Andrea Short and Hazel Platt at the University of Manchester for their help with the SNP array.

References

Barrett, J.C., Fry, B., Maller, J., Daly, M.J., 2005. Haploview: analysis and visualization of LD and haplotype maps. Bioinformatics 21, 263-265.

Cardon, L.R., Bell, J.I., 2001. Association study designs for complex diseases. Nat. Rev. Genet. 2, 91-99.

Cortes, S., Esteves, C., Mauricio, I., Maia, C., Cristovao, J.M., Miles, M., Campino, L., 2012. In vitro and in vivo behaviour of sympatric Leishmania (V.) braziliensis, L. (V.) peruviana and their hybrids. Parasitology 139, 191-199.

Cortes, S., Rolao, N., Ramada, J., Campino, L., 2004. PCR as a rapid and sensitive tool in the diagnosis of human and canine leishmaniasis using Leishmania donovani s.l.-specific kinetoplastid primers. Trans. R. Soc. Trop. Med. Hyg. 98, 12-17.

Criswell, L.A., Pfeiffer, K.A., Lum, R.F., Gonzales, B., Novitzke, J., Kern, M., Moser, K.L., Begovich, A.B., Carlton, V.E., Li, W., Lee, A.T., Ortmann, W., Behrens, T.W., Gregersen, P.K., 2005. Analysis of families in the multiple autoimmune disease genetics consortium (MADGC) collection: the PTPN22 620W allele associates with multiple autoimmune phenotypes. Am. J. Hum. Genet. 76, 561-571.

Davison, L.J., Wallace, C., Cooper, J.D., Cope, N.F., Wilson, N.K., Smyth, D.J., Howson, J.M., Saleh, N., Al-Jeffery, A., Angus, K.L., Stevens, H.E., Nutland, S., Duley, S., Coulson, R.M., Walker, N.M., Burren, O.S., Rice, C.M., Cambien, F., Zeller, T., Munzel, T., Lackner, K., Blakenberg, S., Fraser, P., Gottgens, B., Todd, J.A., Attwood, T., Belz, S., Braund, P., Cooper, J., Crisp-Hihn, A., Diemert, P., Deloukas, P., Foad, N., Erdmann, J., Goodall, A.H., Gracey, J., Gray, E., Williams, R.G., Heimerl, S., Hengstenberg, C., Jolley, J., Krishnan, U., Lloyd-Jones, H., Lugauer, I., Lundmark, P., Maouche, S.,

Moore, J.S., Muir, D., Murray, E., Nelson, C.P., Neudert, J., Niblett, D., O'Leary, K., Ouwehand, W.H., Pollard, H., Rankin, A., Sager, H., Samani, N.J., Sambrook, J., Schmitz, G., Scholz, M., Schroeder, L., Schunkert, H., Syvannen, A.C., Tennstedt, S., 2012. Long-range DNA looping and gene expression analyses identify DEXI as an autoimmune disease candidate gene. Hum. Mol. Genet. 21, 322-333.

Flandin, J.F., Chano, F., Descoteaux, A., 2006. RNA interference reveals a role for TLR2 and TLR3 in the recognition of Leishmania donovani promastigotes by interferon-gamma-primed macrophages. Eur. J. Immunol. 36, 411-420.

Francino, O., Altet, L., Sanchez-Robert, E., Rodriguez, A., Solano-Gallego, L., Alberola, J., Ferrer, L., Sanchez, A., Roura, X., 2006. Advantages of real-time PCR assay for diagnosis and monitoring of canine leishmaniosis. Vet. Parasitol. 137, 214-221.

Gabriel, S.B., Schaffner, S.F., Nguyen, H., Moore, J.M., Roy, J., Blumenstiel, B., Higgins, J., DeFelice, M., Lochner, A., Faggart, M., Liu-Cordero, S.N., Rotimi, C., Adeyemo, A., Cooper, R., Ward, R., Lander, E.S., Daly, M.J., Altshuler, D., 2002. The structure of haplotype blocks in the human genome. Science 296, 2225-2229.

Gramiccia, M., Gradoni, L., 2005. The current status of zoonotic leishmaniases and approaches to disease control. Int. J. Parasitol. 35, 1169-1180.

Hosein, S., Rodriguez-Cortes, A., Blake, D.P., Allenspach, K., Alberola, J., Solano-Gallego, L., 2015. Transcription of Toll-Like Receptors 2, 3, 4 and 9, FoxP3 and Th17 Cytokines in a Susceptible Experimental Model of Canine Leishmania infantum Infection. PLoS One 10, e0140325.

Hostomska, J., Rohousova, I., Volfova, V., Stanneck, D., Mencke, N., Volf, P., 2008. Kinetics of canine antibody response to saliva of the sand fly Lutzomyia longipalpis. Vector Borne Zoonotic Dis. 8, 443-450.

Itoh-Lindstrom, Y., Piskurich, J.F., Felix, N.J., Wang, Y., Brickey, W.J., Platt, J.L., Koller, B.H., Ting, J.P., 1999. Reduced IL-4-, lipopolysaccharide-, and IFN-gamma-induced MHC class II expression in mice lacking class II transactivator due to targeted deletion of the GTP-binding domain. J. Immunol. 163, 2425-2431.

Karmakar, S., Bhaumik, S.K., Paul, J., De, T., 2012. TLR4 and NKT cell synergy in immunotherapy against visceral leishmaniasis. PLoS Pathog 8, e1002646.

Koutinas, A.F., Polizopoulou, Z.S., Saridomichelakis, M.N., Argyriadis, D., Fytianou, A., Plevraki, K.G., 1999. Clinical considerations on canine visceral leishmaniasis in Greece: a retrospective study of 158 cases (1989-1996). Journal of the American Animal Hospital Association 35, 376-383.

Kropf, P., Freudenberg, M.A., Modolell, M., Price, H.P., Herath, S., Antoniazi, S., Galanos, C., Smith, D.F., Muller, I., 2004a. Toll-like receptor 4 contributes to efficient control of infection with the protozoan parasite Leishmania major. Infect. Immun. 72, 1920-1928.

Kropf, P., Freudenberg, N., Kalis, C., Modolell, M., Herath, S., Galanos, C., Freudenberg, M., Muller, I., 2004b. Infection of C57BL/10ScCr and C57BL/10ScNCr mice with Leishmania major reveals a role for Toll-like receptor 4 in the control of parasite replication. J Leukoc Biol 76, 48-57.

Liu, H., Irwanto, A., Fu, X., Yu, G., Yu, Y., Sun, Y., Wang, C., Wang, Z., Okada, Y., Low, H., Li, Y., Liany, H., Chen, M., Bao, F., Li, J., You, J., Zhang, Q., Liu, J., Chu, T., Andiappan, A.K., Wang, N., Niu, G., Liu, D., Yu, X., Zhang, L., Tian, H., Zhou, G., Rotzschke, O., Chen, S., Zhang, X., Zhang, F., 2015. Discovery of six new susceptibility loci and analysis of pleiotropic effects in leprosy. Nat. Genet. 47, 267-271.

Maia, C., Campino, L., 2008. Methods for diagnosis of canine leishmaniasis and immune response to infection. Vet. Parasitol. 158, 274-287.

555 Maia, C., Cardoso, L., 2015. Spread of Leishmania infantum in Europe with dog travelling.
556 Vet. Parasitol. 213, 2-11.
557 Oliva, G., Nieto, J., Foglia Manzillo, V., Cappiello, S., Fiorentino, E., Di Muccio, T., Scalone,
558 A., Moreno, J., Chicharro, C., Carrillo, E., Butaud, T., Guegand, L., Martin, V.,
559 Cuisinier, A.M., McGahie, D., Gueguen, S., Canavate, C., Gradoni, L., 2014. A
560 randomised, double-blind, controlled efficacy trial of the LiESP/QA-21 vaccine in
561 naive dogs exposed to two leishmania infantum transmission seasons. PLoS Negl
562 Trop Dis 8, e3213.
563 Oliva, G., Scalone, A., Foglia Manzillo, V., Gramiccia, M., Pagano, A., Di Muccio, T., Gradoni,
564 L., 2006. Incidence and time course of Leishmania infantum infections examined by
565 parasitological, serologic, and nested-PCR techniques in a cohort of naive dogs
566 exposed to three consecutive transmission seasons. J. Clin. Microbiol. 44, 1318-
567 1322.
568 Palatnik-de-Sousa, C.B., Day, M.J., 2011. One Health: the global challenge of epidemic and
569 endemic leishmaniasis. Parasit Vectors 4, 197.
570 Paltrinieri, S., Solano-Gallego, L., Fondati, A., Lubas, G., Gradoni, L., Castagnaro, M., Crotti,
571 A., Maroli, M., Oliva, G., Roura, X., Zatelli, A., Zini, E., 2010. Guidelines for diagnosis
572 and clinical classification of leishmaniasis in dogs. J. Am. Vet. Med. Assoc. 236,
573 1184-1191.
574 Pinelli, E., Killick-Kendrick, R., Wagenaar, J., Bernadina, W., del Real, G., Ruitenberg, J.,
575 1994. Cellular and humoral immune responses in dogs experimentally and naturally
576 infected with Leishmania infantum. Infect. Immun. 62, 229-235.
577 Purcell, S., Neale, B., Todd-Brown, K., Thomas, L., Ferreira, M.A., Bender, D., Maller, J.,
578 Sklar, P., de Bakker, P.I., Daly, M.J., Sham, P.C., 2007. PLINK: a tool set for whole-
579 genome association and population-based linkage analyses. Am. J. Hum. Genet. 81,
580 559-575.
581 Quilez, J., Martinez, V., Woolliams, J.A., Sanchez, A., Pong-Wong, R., Kennedy, L.J., Quinnell,
582 R.J., Ollier, W.E., Roura, X., Ferrer, L., Altet, L., Francino, O., 2012. Genetic control of
583 canine leishmaniasis: genome-wide association study and genomic selection
584 analysis. PLoS One 7, e35349.
585 Quinnell, R.J., Courtenay, O., Davidson, S., Garcez, L., Lambson, B., Ramos, P., Shaw, J.J.,
586 Shaw, M.A., Dye, C., 2001. Detection of Leishmania infantum by PCR, serology and
587 cellular immune response in a cohort study of Brazilian dogs. Parasitology 122, 253-
588 261.
589 Quinnell, R.J., Kennedy, L.J., Barnes, A., Courtenay, O., Dye, C., Garcez, L.M., Shaw, M.A.,
590 Carter, S.D., Thomson, W., Ollier, W.E., 2003. Susceptibility to visceral leishmaniasis
591 in the domestic dog is associated with MHC class II polymorphism. Immunogenetics
592 55, 23-28.
593 Shaw, S.E., Langton, D.A., Hillman, T.J., 2009. Canine leishmaniosis in the United Kingdom: a
594 zoonotic disease waiting for a vector? Vet. Parasitol. 163, 281-285.
595 Short, A.D., Boag, A., Catchpole, B., Kennedy, L.J., Massey, J., Rothwell, S., Husebye, E.,
596 Ollier, B., 2013. A candidate gene analysis of canine hypoadrenocorticism in 3 dog
597 breeds. J. Hered. 104, 807-820.
598 Short, A.D., Catchpole, B., Kennedy, L.J., Barnes, A., Fretwell, N., Jones, C., Thomson, W.,
599 Ollier, W.E., 2007. Analysis of candidate susceptibility genes in canine diabetes. J.
600 Hered. 98, 518-525.
601 Solano-Gallego, L., Koutinas, A., Miro, G., Cardoso, L., Pennisi, M.G., Ferrer, L., Bourdeau, P.,
602 Oliva, G., Baneth, G., 2009. Directions for the diagnosis, clinical staging, treatment
603 and prevention of canine leishmaniosis. Vet. Parasitol. 165, 1-18.

Solano-Gallego, L., Riera, C., Roura, X., Iniesta, L., Gallego, M., Valladares, J.E., Fisa, R., Castillejo, S., Alberola, J., Ferrer, L., Arboix, M., Portus, M., 2001. Leishmania infantum-specific IgG, IgG1 and IgG2 antibody responses in healthy and ill dogs from endemic areas. Evolution in the course of infection and after treatment. Vet. Parasitol. 96, 265-276.

Solano-Gallego, L., Villanueva-Saz, S., Carbonell, M., Trotta, M., Furlanello, T., Natale, A., 2014. Serological diagnosis of canine leishmaniosis: comparison of three commercial ELISA tests (Leiscan, ID Screen and Leishmania 96), a rapid test (Speed Leish K) and an in-house IFAT. Parasit Vectors 7, 111.

Stanford, S.M., Bottini, N., 2014. PTPN22: the archetypal non-HLA autoimmunity gene. Nat Rev Rheumatol 10, 602-611.

Utsunomiya, Y.T., Ribeiro, E.S., Quintal, A.P., Sangalli, J.R., Gazola, V.R., Paula, H.B., Trinconi, C.M., Lima, V.M., Perri, S.H., Taylor, J.F., Schnabel, R.D., Sonstegard, T.S., Garcia, J.F., Nunes, C.M., 2015. Genome-Wide Scan for Visceral Leishmaniasis in Mixed-Breed Dogs Identifies Candidate Genes Involved in T Helper Cells and Macrophage Signaling. PLoS One 10, e0136749.

Vlkova, M., Rohousova, I., Drahota, J., Stanneck, D., Kruedewagen, E.M., Mencke, N., Otranto, D., Volf, P., 2011. Canine antibody response to Phlebotomus perniciosus bites negatively correlates with the risk of Leishmania infantum transmission. PLoS Negl Trop Dis 5, e1344.

Zhang, X., Hong, X., Deng, G., Bai, X., 2007. Single nucleotide polymorphisms and functional analysis of class II transactivator (CIITA) promoter IV in persistent HBV infection. J. Clin. Virol. 40, 197-201.

Figure Legends

Figure 1

Leishmania diagnostic summary for Study 1 clinical cases and controls. Clinical case (n=62) and control (n=101) dogs were tested for *Leishmania* antibodies by ELISA and *Leishmania* DNA in the peripheral blood was assessed by qPCR. +/- = positive/negative result, Sero = ELISA result, qPCR = qPCR result. Cases are indicated by black bars and controls are indicated by grey bars.