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Original Article

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.

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). *L. 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

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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 (see Appendix: Supplementary material). 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 Appendix: Supplementary Table 1. Dogs in studies A ($n = 30$) and B ($n = 17$) were challenged with 1×10^6 promastigotes, dogs in study C ($n = 23$) were challenged with 5×10^7 promastigotes and dogs in study D ($n = 20$) were challenged with 2×10^8 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 (Appendix: 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 1:12,800 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 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 $MgCl_2$ (2.5 mM 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