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Research paper

Leishmania infantum-specific IFN-γ production in stimulated blood from dogs with clinical leishmaniosis at diagnosis and during treatment

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ABSTRACT

There is limited data regarding *Leishmania infantum* specific T cell mediated immunity in naturally infected sick dogs at the time of diagnosis and during anti-*Leishmania* treatment. Our aim was to investigate the kinetics of *L. infantum* specific IFN-γ production in dogs with leishmaniosis at the time of diagnosis and during treatment and to correlate it with specific *L. infantum* antibodies, blood parasitemia and clinicopathological findings. Thirty-four dogs were diagnosed with leishmaniosis based on physical examination, routine laboratory tests and *L. infantum*-specific antibody levels by quantitative ELISA. Heparinized whole blood was stimulated with *L. infantum* soluble antigen (LSA) and concanavalin A (ConA) and incubated for 5 days. IFN-γ concentration was evaluated in supernatants of stimulated blood using a commercial sandwich ELISA. *Leishmania* real-time PCR was also performed for assessing blood parasitemia. Dogs were treated with meglumine antimoniate and allopurinol.

Sixteen dogs were classified as IFN-γ non-producers after LSA stimulation (mean ± SD: 0 ± 0 pg/mL) and 18 dogs as IFN-γ producers (mean ± SD: 2885.3 ± 4436.1 pg/mL) at the time of diagnosis ($P < 0.0001$). IFN-γ non-producers were classified in a more severe clinical staging than IFN-γ producers that presented a mild to moderate clinical staging ($P = 0.03$). In the IFN-γ non-producer group, production of IFN-γ after LSA stimulation was significantly increased during treatment especially at day 365 ($P = 0.018$) together with clinical improvement when compared with day 0. In contrast, IFN-γ producers maintained their IFN-γ production after LSA stimulation and no statistically significant changes were found during treatment follow-up. At diagnosis, IFN-γ non-producers showed a significantly higher blood parasitemia *versus* IFN-γ –producers ($P = 0.005$). IFN-γ non-producers drastically reduced blood parasitemia to minimum values at day 365 when compared with day 0 ($P = 0.017$). No significant differences were found at day 365 in blood parasitemia of IFN-γ producers compared to pre-treatment. At diagnosis, *L. infantum* specific antibodies were higher in IFN-γ non-producers than IFN-γ producers ($P = 0.014$). A marked reduction of antibody levels was found at day 365 when compared with day 0 in IFN-γ non-producers ($P = 0.005$) and producers ($P = 0.001$). These results demonstrate that IFN-γ concentration increases with long-term anti-*Leishmania* treatment together with clinical improvement in dogs that do not produce IFN-γ at diagnosis. Together with clinical recovery, reduction in blood parasitemia and *L. infantum* specific antibodies, tracking IFN-γ concentration could constitute an important prognostic tool for immune monitoring in CanL.

Abbreviations: CBC, complete blood cell count; ConA, concanavalin A; DNA, deoxyribonucleic acid; ELISA, enzyme-linked immunosorbent assay; EU, ELISA units; IFAT, indirect immunofluorescence technique; IFN-γ, interferon-gamma; IL-10, interleukin-10; IL-4, interleukin 4; LSA, *L. infantum* soluble antigen; LST, Leishmanin skin test; NO, nitric oxide; PBMC, peripheral blood mononuclear cells; PBS-Tween, phosphate buffer solution-Tween; PD-1, programmed cell death protein-1; P-MAPA, protein aggregate magnesium-ammonium phospholinoleate-palmitoleate anhydride; RPMI-1640, Roswell Park Memorial Institute 1640 medium; RT-PCR, real time PCR; SD, standard deviation; Th1, type 1 T helper lymphocytes; Th2, type 2 T helper lymphocytes; TNFα, tumor necrosis factor-alpha; UPC, urinary protein/creatinine ratio; WBA, whole blood stimulation assay

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1. Introduction

Canine leishmaniosis (CanL) is caused by protozoa *Leishmania infantum* (Baneth et al., 2008), which is an intracellular parasite of the mononuclear phagocyte system being transmitted by *Phlebotomus* sand fly bites (Akhoundi et al., 2016). This infection is endemic and affects dogs and humans in the Mediterranean basin (Baneth et al., 2008; Gramiccia and Gradoni, 2005), China (Zhao et al., 2016) and South America (Dantas-Torres, 2009), and is an emergent disease in North America (Duprey et al., 2006) and in the northern latitudes of Europe (Espejo et al., 2015; Ready, 2010). CanL can manifest as chronic subclinical infection, self-limiting disease, or non-self-limiting disease which can be fatal. In addition, the severity of this disease ranges from mild to very severe. According to this variability, a clinical staging system has been proposed by the LeishVet group (Solano-Gallego et al., 2017, 2009).

The most common clinical signs in dogs with moderate to very severe disease include cutaneous lesions such as exfoliative dermatitis, onychogryphosis, ulcerations, generalized lymphadenomegaly and lesions derived from immune-complexes deposition such as uveitis, vasculitis or glomerulonephritis. Frequent clinicopathological abnormalities are mild non-regenerative anemia, hypoalbuminemia, hyperglobulinemia and mild proteinuria. Mild disease is commonly diagnosed with mild cutaneous lesions such as papular dermatitis (Lombardo et al., 2014; Ordeix et al., 2005) or with solitary lymphadenomegaly without clinicopathological abnormalities (Solano-Gallego et al., 2011).

The diagnosis of clinical leishmaniosis is challenging due to the wide spectrum of its clinical and immunological manifestations. Laboratory diagnostic methods commonly used in the clinical practice for this disease include serology to detect *L. infantum* antibodies. Serological methods include the indirect immunofluorescence technique (IFAT) and enzyme-linked immunosorbent assay (ELISA) currently considered the most sensitive tests. Cytology, histopathology and real-time polymerase chain reaction (RT-PCR) are used to demonstrate infection in tissues (Maia and Campino, 2008).

The most standardized treatment for the disease consists of using several drugs with different mechanisms of action, which reduce *Leishmania* infection. The combination of meglumine antimoniate or miltefosine, and long term treatment with allopurinol, is the most used and effective treatment protocol in clinical practice (Noli and Saridomichelakis, 2014).

It is well known that the clinical manifestations and outcome of infection in CanL is a consequence of complex interactions between the parasite and the genetic and immunological background of the dog (Hosein et al., 2017). The progression of infection to active disease in susceptible dogs is characterized by a marked humoral response, a depression of the cellular immune response against the parasite and the appearance of a full array of clinical signs and/or clinicopathological abnormalities. In the opposite spectrum, resistant dogs lack clinical signs or clinicopathological abnormalities, develop low levels of anti-*Leishmania* antibodies and parasite loads, and a strong *in vitro* lymphocyte proliferative response (Carrillo and Moreno, 2009; Solano-Gallego et al., 2016b). The resistant profile in healthy infected dogs is due to the onset of a strong T helper 1 (Th1)-like response against the parasite, mediated principally by a high expression of interferon gamma (IFN-γ) and tumor necrosis factor-α (TNF-α). Conversely, a susceptible profile and the establishment of clinical disease is mediated by a Th2-like immune response associated with anti-inflammatory cytokines such as interleukin- 4 (IL-4) and IL-10 together with different degrees of detection of IFN-γ in a low proportion of dogs (Baneth et al., 2008; Barbieri, 2006; Boggiatto et al., 2010; Esch et al., 2013).

Different methods for the evaluation of specific T cell mediated immunity have been investigated in CanL although standardization is lacking. One of the most sensitive methods is the leishmanin skin test (LST) based on a delayed hypersensitivity reaction. LST specifically detects cellular immunity against the parasite *in vivo* in the dog, especially when illness is not present and is frequently used in epidemiological investigations in CanL endemic regions (Cardoso et al., 1998; Fernandez-Bellon et al., 2005; Solano-Gallego et al., 2000). Other methods like the IFN-γ cytopathic effect inhibition bioassay, detection of IFN-γ transcripts and lymphocyte proliferation assays have been tested, with impaired results (Fernandez-Bellon et al., 2005; Hosein et al., 2017).

Another example of measurement of T cell-mediated immunity in leishmaniosis is quantifying specific IFN-γ production in *Leishmania* stimulated whole blood or peripheral blood mononuclear cells (PBMC). This assay is commonly used to evaluate the immunogenicity of *Leishmania* vaccines (Carson et al., 2009; Costa-Pereira et al., 2015). However, limited information is available regarding *L. infantum* specific IFN-γ production in stimulated blood in sick dogs with different degrees of disease at the time of diagnosis and during treatment and clinical cure (Boggiatto et al., 2010; Strauss-Ayali et al., 2005). A recent study performed by our group, demonstrated that IFN-γ producers sick dogs were associated with lower antibody levels, parasite load and milder disease when compared with IFN-γ non-producers (Solano-Gallego et al., 2016b). We hypothesize that IFN-γ non-producer dogs might recover IFN-γ production when effective anti-*Leishmania* treatment is provided and clinical improvement is achieved. In contrast, it is likely that IFN-γ producer sick dogs might maintain or slightly increase their initial IFN-γ concentration.

The aim of this study was to investigate the kinetics of *L. infantum* specific IFN-γ production in blood from dogs with clinical leishmaniosis (IFN-γ producers *versus* IFN-γ non-producers) at the time of diagnosis and during long term standard anti-*Leishmania* treatment, and to correlate with clinicopathological, parasitological and serological data.

2. Materials and methods

2.1. Dogs

Five mixed breed and 29 pure breed sick dogs (age range between 5 and 153 months) from an endemic area of CanL (Catalonia, Spain) were prospectively enrolled in this study from January 2014 to January 2016. Breeds of sick dogs are described in Supplementary Table 1. All sick dogs attended three veterinary centres: *Hospital Clinic Veterinari* of Autonomous University of Barcelona (Bellaterra, Spain), ARS Veterinària (Barcelona, Spain) and Consultori Montsant (Tarragona, Spain). A full physical examination and routine laboratory tests were performed in all dogs. All dogs were diagnosed with leishmaniosis based on two-fold serial dilution ELISA antibody levels as described previously (Solano-Gallego et al., 2016a) and/or by observation of amastigotes on cytological and/or histopathological evaluation in skin and/or lymph nodes.

Ten mL of blood sample were taken by jugular or cephalic venipuncture and in ethylenediaminetetraacetic acid (EDTA), heparinized and serum sterile tubes for routine haematology [Siemens Advia 120 Haematology System (Siemens Healthcare GmbH, Germany)], serum biochemistry [Olympus AU400 Chemystry Analyzer (CLIAwaived, USA)] and serum electrophoresis [Hydrasys® (Sebia Electrophoresis, USA)]. Blood parasite quantification by *Leishmania* RT-PCR and serology for the detection of *L. infantum* antibodies at the time of diagnosis (day 0) and during treatment at days 30, 180 and 365 were also performed as previously described (Solano-Gallego et al., 2016a).

Urine samples from all dogs were mostly taken by cystocenthesis for urinalysis and for urinary protein creatinine ratio (UPC). All dogs were classified into clinical stages following the Leishvet guidelines according to their clinicopathological findings and serological results at the time of diagnosis (Solano-Gallego et al., 2009).

We prospectively evaluated the possibility of coinfection in the dogs that presented clinical signs and/or clinicopathological abnormalities that were highly compatible with other infectious diseases at discretion

of the clinician in charge of the case at the time of diagnosis and if needed during treatment. This was mainly assessed in dogs with leukopenia, pancytopenia, thrombocytopenia or extremely marked hyperproteinemia with hypergammaglobulinemia. The results by the Snap 4Dx (IDEXX Laboratories) and by *Ehrlichia/Anaplasma/Babesia* PCRs were negative for all the dogs tested for co-infection. In addition, it is important to highlight that a full CBC with a blood smear examination was performed by a specialized technician in all dogs studied at the time of diagnosis and during treatment follow-up (day 30, day 180 and day 365). No hemoparasites were visualized by blood smear microscopy in any of the dogs included in the present study.

All blood samples with the exception of those from the three sick dogs were retrospectively screened in duplicates for the presence of *E. canis* DNA using real-time PCR. Briefly, a 123 base-pair (bp) segment of the 16S ribosomal RNA gene of *E. canis* was amplified using primers E.c 16S-fwd (5′-TCGCTATTAGATGAGCCTACGT-3′) and E.c 16S-rev (5′-GAGTCTGGACCGTATCTCAG-3′) as previously described (Peleg et al., 2010). All dogs resulted negative.

2.2. Anti-Leishmania treatment

Treatment was instituted as follows: 75–100 mg/kg meglumine antimoniate (Glucantime®) injected subcutaneously by the owner once a day for a 28 days and allopurinol given orally twice a day for up 12 months at 10 mg/kg. As mentioned above, treatment monitoring was scheduled on days 30, 180 and 365 for evaluation of clinical status (complete physical examination as well as evaluation of the same clinicopathological parameters as described in subsection 2.1).

2.3. Blood DNA extraction and Leishmania RT-PCR

DNA was obtained from 400 µL of EDTA whole blood using GenElute™ Blood Genomic DNA extraction Kit, (Sigma Aldrich, Missouri, USA) following the manufacturer's instructions with slight modifications. Forty µL of proteinase K were added to all samples. Blood from a clinically healthy non-infected dog was used as a negative control for eventually DNA contamination in every DNA extraction performed.

RT-PCR was performed with an absolute quantification (Solano-Gallego et al., 2016a). Briefly, PCR mix reaction was obtained with 4 µL of DNA, 10 µL of Taqman Fast Advanced Master Mix (Life Technologies™, California, USA), 1 µL of *Leishmania* primers and probes [Custom Taqman Gene Expression Assay, (Life Technologies™, California USA)] or 1 µL of primers and probes [(Eukaryotic 18S rRNA Endogenous Control (VIC™/MGB Probe, Primer Limited)] and 5 µL of H_2O. PCR reaction was run in duplicates for each sample.

In order to consider the PCR assay validated, a positive control for *Leishmania*, a negative control from a non-infected clinically healthy dog and a blank (well with no DNA sample) were included in all plates.

RT-PCR was carried out in a QuantStudio Flex™ 7 Real-Time PCR system (Life Technologies™, California USA). Thermal cycling profile consist of 50 °C 2 min in order to activate the enzyme amperase and 20 s at 95 °C followed by 40 cycles of 1 s at 95 °C and 20 s at 60 °C. Absolute quantification was calculated by the interpolation of the samples to the standard curve obtained from a negative sample spiked with different quantities of *Leishmania* promastigotes. Final results were classified as negative (0 parasites/mL), low positive (< 10 parasites/mL), medium positive (10–100 parasites/mL, high positive (100–1000 parasites/mL) or very high positive (> 1000 parasites/mL) (Solano-Gallego et al., 2016a).

2.4. ELISA for specific L. infantum antibody detection

The in-house ELISA was performed on sera of all dogs as previously described (Solano-Gallego et al., 2016a). Briefly, the samples were diluted to 1:800 in phosphate buffer solution (PBS) with 0.05%Tween 20 (Sigma Aldrich, Missouri, USA) containing 1% dry milk and incubated in *L. infantum* antigen-coated plates (20 µg/mL) for 1 h at 37 °C. Then, the plates were washed three times with PBS-Tween and once with PBS and incubated with Protein A conjugated to horseradish peroxidase (Invitrogen, California, USA) at 1:30,000 dilution in 0.05% Tween 20 in phosphate-buffered saline (PBS) with 1% of dry milk for 1 h at 37 °C. After that, the plates were washed again as described above. The plates were developed by adding the substrate solution *o*-phenylenediamine and substrate buffer SIGMA*FAST* OPD (Sigma Aldrich, Missouri, USA). The reaction was stopped with 50 µL of 2.5 M H_2SO_4. Absorbance values were read at 492 nm by an automatic reader (ELISA Reader Anthos 2020). All plates included the serum from a sick dog with confirmed infection as positive control and serum from a healthy dog as a negative control. All samples were analysed in duplicate. The result was quantified as ELISA units (EU) related to a positive canine serum used as a calibrator and arbitrarily set at 100 EU.

All samples with an optical density (OD) equal or higher than three were repeated using a two-fold serial dilution ELISA. Sera two-fold dilutions were started at 1:800 and continued for 9–11 further dilutions for all time points studied for each dog (days 0, 30, 180 and 365). All samples were analysed on the same day and in the sample plate to avoid variability. The result was quantified as EU related to a calibrator set at 100 EU, with an OD value of one at the 1:800 dilution. The mean values of the dilution at which the OD were close to one was chosen for the calculation of the positivity% using the following formula: (Sample OD/Calibrator OD) x 100x dilution factor.

Sera were classified as: very high positive, when having equal or higher than 40000 EU; high positive, when having equal or higher than 9000 EU and less than 40000 EU; medium positive, when having equal or higher than 500 EU and less than 9000 EU; low positive, when having lower than 500 EU and equal or higher than 100 EU; very low positive, when having lower than 100 EU and equal or higher than 35 EU. Sera lower than 35 EU, were classified as negative (Solano-Gallego et al., 2016a).

2.5. Whole blood stimulation assay (WBA)

Five hundred µL of heparinized blood were diluted in each well immediately after sample collection at a ratio of 1:10 with a volume of 4.5 mL of RPMI 1640 with stable glutamine and 25 mM hepes (Biowest® Kansas, USA) supplemented with 100 U/mL of penicillin, 100 µg/mL streptomycin (Life Technologies™, California, USA) and 10% of fetal bovine serum premium South America origin (Biowest® Kansas, USA).

Three different conditions were established: 1) medium alone 2) medium with soluble *L. infantum* antigen (LSA) at a concentration of 10 µg/mL provided by Dr. Cristina Riera (*L. infantum* antigen 5 mg/mL, *Facultat de Farmacia, Universitat de Barcelona*) and 3) medium with mitogen concanavalin A (ConA) (100 mg Medicago® Uppsala, Sweden) at a concentration of 10 µg/mL. LSA was prepared as described with slight modifications (Carrillo et al., 2015). Washed, cultured *L. infantum* (MHOM/FR/LEM75/MON1) at a concentration of 1×10^9/ ml in PBS was frozen and thawed three times and then centrifuged (8.000*g*, 30 min, 4 °C). The supernatant was collected and protein concentration was determined.

Blood samples were incubated in twelve-well flat bottom plastic culture plates (Costar® Corning, NY, USA) for 5 days at 37 °C in 5% CO_2 environment. Then, blood was collected in sterile tubes and centrifuged at 300*g* for 10 min. The supernatant was stored frozen at − 80 °C until used (Solano-Gallego et al., 2016b).

2.6. IFN-γ measurement

IFN-γ concentration was determined from the supernatant obtained by WBA. This cytokine was evaluated following the manufacturer's instructions (DuoSet® ELISA by Development System R & D™, Abingdon, UK) using 96 well cell flat bottom plates (Costar®Corning, NY, USA).

Slight modifications were performed. Standard curve for IFN-γ started at 8000 pg/mL and two-fold dilutions were made until 62.5 mg/mL. Supernatants containing ConA were diluted 1:1 with reagent diluent provided by the manufacturer.

All the samples were measured in duplicates in all ELISAs. The OD was determined by automatic absorbance microplate reader (Anthos 2020) at wavelength of 450 nm. The standard curve and concentration quantification for IFN-γ was calculated using a four parameter logistic-curve provided by MyAssays program (http://www.myassays.com/). Plates were repeated when the R^2-value of standard curve was below 0.98. All samples from each dog at the time of diagnosis and during treatment follow-up were analysed on the same plate.

Sixteen dogs were classified as non-IFN-γ producers when *L. infantum* specific IFN-γ concentration was undetectable. The remaining dogs were classified as IFN-γ producers when *L. infantum* specific IFN-γ concentrations were higher than 62.5 pg/mL (Solano-Gallego et al., 2016b).

2.7. Statistical analysis

The statistical analysis was performed using the SPSS 17.0 for Windows software (SPSS Inc., USA). A non-parametric Mann-Whitney *U* test was used to compare unpaired quantitative variables. A non-parametric Wilcoxon signed-rank test was used to compare paired continuous variables. A Fisher's exact test was used to compare sex and the non-parametric Mann-Whitney *U* test was used to compare age among groups. Significant differences were considered at a 5% significance level ($P < 0.05$). Graphs were created using the excel GraphPad Prism 6 (GraphPad Software, La Jolla, CA, USA).

2.8. Ethics

Dog owners signed written informed consent prior to sampling them. All the sampling and analytics were performed as part of the veterinary clinical routine. Residual blood samples were used in this study.

3. Results

3.1. Clinical staging and specific L. infantum IFN-γ response classification at the time of diagnosis

A total of 34 dogs with leishmaniosis were included in this study, including 14 females and 20 males. The median age was 54 months with a range of five months to twelve years at the time of diagnosis. At the time of diagnosis, all dogs presented several and typical clinicopathological findings of disease and mainly medium to high positive antibody levels. Dogs were classified in four clinical stages: stage I-mild disease (n = 1), stage II-moderate disease (stage IIa, n = 20 and stage IIb, n = 6), stage III-severe disease (n = 6) and stage IV-very severe disease (n = 1)(Solano-Gallego et al., 2009).

Further classification was made in order to differentiate IFN-γ non-producer and IFN-γ producer dogs. IFN-γ non-producer dogs (n = 16) included 12 males and 4 females, all were pure breed dogs except two (Supplementary Table 1), with a median of age of 44 months. The IFN-γ producer group (n = 18) included 8 males and 10 females, with 14 pure breed and 3 mixed breed dogs (Supplementary Table 1), and a median age of 64 months. Although, dogs under study presented a wide range of age (between 5 and 153 months), no statistical differences were found regarding sex and age between the groups.

Clinical staging based on IFN-γ classification is shown in Fig. 1.

The majority of IFN-γ producer dogs (78%) were classified in stages I or IIa while only 43% of IFN-γ non-producer dogs were classified in stage IIa and none were classified in stage I ($P = 0.03$) (Fig. 1).

3.2. IFN-γ concentration at time of diagnosis and during treatment follow-up

At day 0, supernatants from LSA stimulated whole blood of IFN-γ producer dogs presented significantly higher concentrations of IFN-γ with a mean ± SD of 2885.3 ± 4436.1 pg/mL when compared with IFN-γ non producers, which showed a mean ± SD of 0 ± 0 pg/mL ($P < 0.0001$) (Fig. 2a). Levels of IFN-γ of ConA stimulated blood from IFN-γ non-producers (mean ± SD: 4874.6 ± 4221.8 pg/mL) exhibited statistically significant lower concentrations ($P = 0.042$) than IFN-γ producers group (mean ± SD: 11593.0 ± 9278.3 pg/mL).

During a year of treatment follow-up, seven out of 16 (43%) IFN-γ non-producer dogs significantly increased their production, reaching the highest point at day 365 (mean ± SD: 1744.3 ± 4201.1 pg/mL) in response to LSA stimulation ($P = 0.018$). In addition, those seven dogs also reduced antibody levels and parasite load by RT-PCR (Fig. 2b,c). Moreover, at day 30 post treatment, two dogs started producing low levels of IFN-γ (mean ± SD: 12.9 ± 34.3 pg/mL), which was not as statistically significant increase. A significantly higher production of IFN-γ ($P = 0.043$) at day 180 was also found, represented by five out of 16 dogs (mean ± SD: 1214.1 ± 3198.8) when compared with day 0 (mean ± SD of 0 ± 0 pg/mL) (Fig. 2a). However, despite a long treatment period, some animals from the IFN-γ non-producer group did not produce specific *L. infantum* IFN-γ (day 180; n = 11) and (day 365; n = 7).

No statistical differences in the days post-treatment were found in IFN-γ producer dogs regarding their IFN-γ concentration during follow-up.

3.3. Evaluation of specific L. infantum antibodies at diagnosis and during treatment follow-up

The kinetics of antibody levels over time of both groups is shown in Fig. 2b.

At time of diagnosis, IFN-γ non-producer dogs presented significantly higher antibody levels (mean ± SD: 13754.4 ± 17856.2 EU) than IFN-γ producers (mean ± SD: 2509.5 ± 4011.1 EU) ($P = 0.014$).

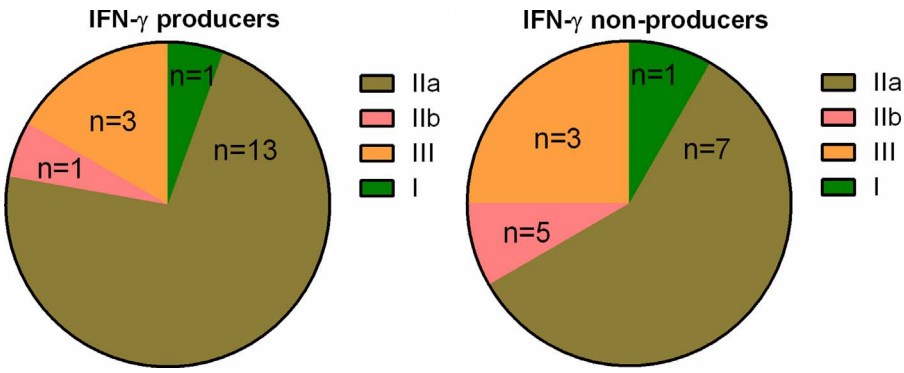

Fig. 1. Distribution of dogs according to IFN-γ production in *ex vivo* blood after LSA stimulation and their clinical staging (Solano-Gallego et al., 2011).

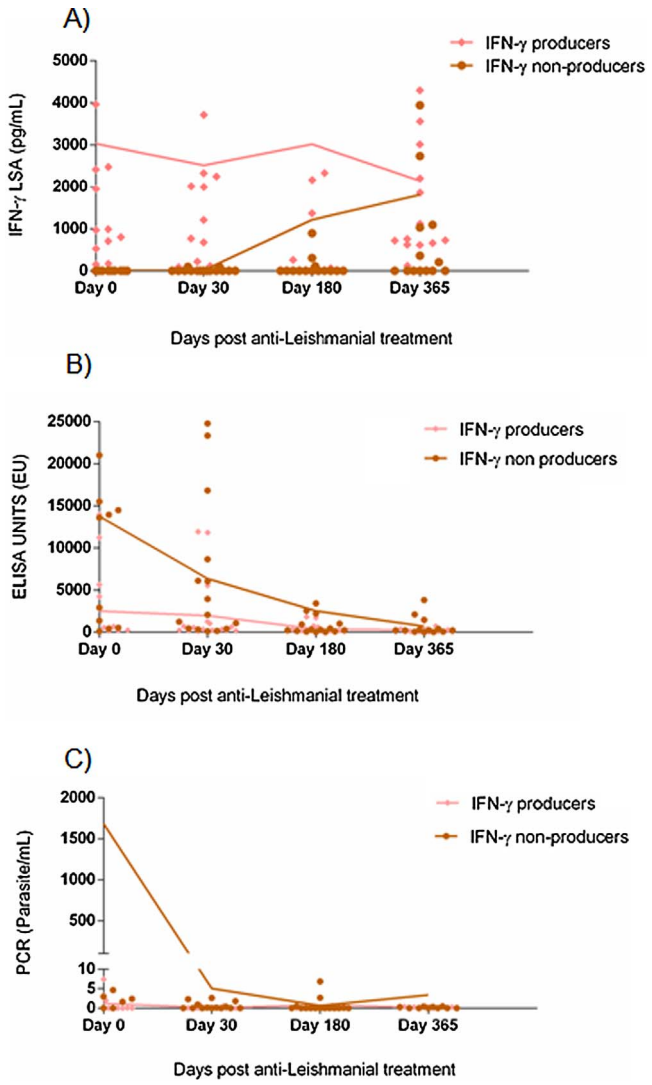

Fig. 2. Comparative results during treatment follow-up (days 0, 30, 180 and 365). a) specific LSA IFN-γ production of IFN-γ producers *versus* IFN-γ non-producers, b) specific-*L. infantum* antibody response of IFN-γ producers *versus* IFN-γ non-producers and c) Parasite load of IFN-γ producers *versus* IFN-γ non-producers.

Interestingly, IFN-γ non-producer dogs drastically decreased their antibody levels in the first check-up. At day 30, antibody levels in the IFN-γ non-producer group (mean ± SD: 6369.3 ± 8473.4 EU) were significantly lower than levels at time of diagnosis (P = 0.02). Furthermore, antibody levels were markedly lower at day 180 (mean ± SD: 2539.3 ± 7016.2 EU, P = 0.0001) and at day 365 reaching a mean ± SD of 702.9 ± 1125.0 EU when compared with day 0 (P = 0.005).

On the other hand, antibody levels on IFN-γ producer dogs were significantly lower at day 365 (mean ± SD: 247.4 ± 342.5) when compared with day 0 (P = 0.001). Furthermore, in this group of dogs, the results from ELISA dilutions were significantly lower (P = 0.022) at day 30 (mean ± SD: 1970.6 ± 3812.8) and also at day 180 (P = 0.001) with a mean ± SD: 387.4 ± 557.2 when compared with day 0 (mean + SD: 2509.5 ± 4011.1) in conjunction with clinical improvement.

Despite an overall reduction of the antibodies showed by both groups, only three dogs (IFN-γ producer (n = 1) and IFN-γ non-producer (n = 2) at time of diagnosis) were seronegative at day 180 (9%) and four dogs (IFN-γ producer (n = 2) and IFN-γ non- producer (n = 2) at time of diagnosis) presented negative results for detection of antibodies level at day 365 (12%). The rest of the dogs presented very low to medium positive antibodies level at the end of the follow up. No dogs remained high or very high positive.

3.4. Blood parasitemia at diagnosis and during treatment follow-up

The results of blood parasitemia are shown in Fig. 2c. Overall, only 57% of all studied dogs were positive by *L. infantum* RT-PCR at the time of diagnosis. The majority of IFN-γ non-producer dogs (81%) were found positive by blood RT-PCR while only 35% of IFN-γ producer dogs resulted positive. This difference was statistically significant (P = 0.01). All positive RT-PCR dogs were classified as medium or low positive, with only one exception, a dog with a very high parasitemia.

As expected, when parasite load was measured at the time of diagnosis, IFN-γ non-producers group had a significantly higher blood parasite load (mean ± SD: 1683.9 ± 6474.4 parasites/mL) than IFN-γ producer dogs (mean ± SD: 1.2 ± 2.1 parasites/mL) (P = 0.005).

Treatment was effective in reducing the parasite load, especially in IFN-γ non-producer dogs. At day 30, the parasite load was significantly reduced with a mean ± SD of 5.0 ± 17.3 parasites/mL (P = 0.028) and a progressive reduction was also observed at day 180 (mean ± SD: 0.6 ± 1.8 parasites/mL) (P = 0.003) until day 365 (mean ± SD: 0.1 ± 0.2 parasites/mL, P = 0.017).

No significant differences were observed in the parasite load in IFN-γ producer dogs during treatment follow-up (Fig. 2c).

It is important to point out that the kinetics of RT-PCR values and the levels of antibodies followed a similar trend as in both groups, a RT-PCR value of close to zero was reached.

3.5. Clinicopathological findings at diagnosis and during treatment follow-up

The results of clinicopathological findings are listed in Table 1. All dogs improved clinically during treatment follow-up and the laboratory abnormalities decreased during the treatment period.

Gamma-globulins in IFN-γ non-producer dogs markedly decreased over treatment follow-up, from a mean ± SD of 3.2 ± 2.5 g/dL at time of diagnosis to 0.7 ± 0.4 g/dL at day 365 (P = 0.01). The same was detected in IFN-γ producer dogs, but to a lesser extent, from a mean ± SD of 1.3 ± 1.5 g/dL at day 0–0.5 ± 0.1 g/dL (P = 0.021) at day 365. Only one dog from the IFN-γ producer group showed an increase in gamma-globulins at day 30, which normalized at 180 and 365 days.

Similar to the gamma-globulins, total proteins also exhibited a significant reduction in levels over the treatment period in both groups, especially at day 180 and 365 (P < 0.0001). All IFN-γ producer dogs normalized total proteins levels at day 30, with one exception.

A significant increase in albumin levels was noted along treatment follow-up in both groups studied (Table 1).

A significant improvement was also detected in haematological parameters. The haematocrit and haemoglobin values reached normal values in average at the end of treatment period in both groups (Table 1). Among the IFN-γ non-producer dogs, only one dog out of 16 maintained low haematological parameters along the treatment and the rest of sick dogs had normal values at day 180 and 365. The IFN-γ-producer dogs reach normal parameters from day 30 onwards.

UPC ratio showed significant differences between day 0 and day 30 in IFN-γ non-producer dogs, from mean ± SD 1.0 ± 1.8, clearly proteinuric to 0.3 ± 0.2, borderline result (P = 0.008). IFN-γ producer dogs did not decrease their average UPC values. In addition, creatinine and urea, evaluated as well, did not show significant changes in both groups.

4. Discussion

Previous investigations on CanL demonstrated the importance of the specific IFN-γ response at diagnosis (Solano-Gallego et al., 2016b),

Table 1
Results of clinicopathological findings based in IFN-γ production in dog groups at the time of diagnosis and during treatment follow-up.

Follow up time (days)	IFN-γ producers (mean ± standard deviation) (n = 18)						IFN-γ non-producers (mean ± standard deviation) (n = 16)					
	Haematocrit (%)	Haemoglobin (g/dL)	Total protein (g/dL)	Albumin (g/dL)	Gamma globulins (g/dL)	UPC	Haematocrit (%)	Haemoglobin (g/dL)	Total protein (g/dL)	Albumin (g/dL)	Gamma globulins (g/dL)	UPC
0	39.01 ± 5.97[r]	13.75 ± 2.01[w]	7.20 ± 1.59[h]	2.87 ± 0.45	1.30 ± 1.50[c]	0.90 ± 1.43	32.68 ± 9.98[p]	11.40 ± 4.24[t]	8.93 ± 2.45[f]	2.28 ± 0.55[j]	3.27 ± 2.50[a]	1.07 ± 1.80[m]
30	42.78 ± 6.48	14.78 ± 2.17	6.79 ± 0.86	2.81 ± 0.49	1.07 ± 0.88	0.79 ± 1.35	39.85 ± 7.13	13.51 ± 2.72	7.73 ± 2.30	3.04 ± 1.93	1.58 ± 1.26	0.31 ± 0.27
180	46.65 ± 7.01	16.23 ± 2.32	6.59 ± 0.34	3.34 ± 0.42	0.57 ± 0.14	0.75 ± 1.43	43.13 ± 5.73	15.14 ± 2.58	6.82 ± 1.88	3.05 ± 0.64	1.12 ± 1.70	0.46 ± 0.52[n]
365*	47.23 ± 8.35[q]	16.56 ± 2.75[v]	6.48 ± 0.42[g]	3.42 ± 0.29[k]	0.54 ± 0.11[d]	0.71 ± 1.10	45.09 ± 6.90[o]	16.14 ± 2.43[s]	6.68 ± 0.47[e]	3.13 ± 0.44[i]	0.77 ± 0.40[b]	0.30 ± 0.29

UPC: urinary protein: creatinine ratio.
Results for IFN-γ non-producer gamma-globulins day 365[a] < IFN-γ non-producer gamma-globulins day 0[b] (P = 0.01); IFN-γ producer gamma-globulins day 365[c] < IFN-γ producer gamma globulins day 0[d] (P = 0.021).
Results for IFN-γ non-producer total proteins day 365[e] < IFN-γ non-producer total proteins day 0[f] (P = 0.01); IFN-γ producer total proteins day 365[g] < IFN-γ producer total proteins day 0[h] (P = 0.087).
Results for IFN-γ non-producer albumin day 365[i] > IFN-γ non-producer albumin day 0[j] (P = 0.005); IFN-γ producer albumin day 365[k] > IFN-γ producer albumin day 0[l] (P = 0.001).
Results for IFN-γ non-producer UPC day 0[m] > IFN-γ non-producer UPC day 180[n] (P = 0.031).
Results for IFN-γ non-producer haematocrit day 365[o] > IFN-γ non-producer haematocrit day 0[p] (P = 0.015); IFN-γ producer haematocrit day 365[q] > IFN-γ producer haematocrit day 0[r] (P = 0.008).
Results for IFN-γ non-producer haemoglobin day 365[s] > IFN-γ non-producer haemoglobin day 0[t] (P = 0.028); IFN-γ producer haemoglobin day 365[v] > IFN-γ producer haemoglobin day 0[w] (P = 0.008).
* The number of dogs IFN-γ non-producers was 13.

however limited data on parasite specific IFN-γ production during anti-*Leishmania* treatment is available in dogs (Manna et al., 2008b). In this study, we investigated the cellular immune responses of dogs with clinical leishmaniosis at different clinical stages of the disease during a standard 6–12 months treatment. Here, we evaluated clinical parameters, antibodies levels, blood parasitemia and specific *L. infantum* IFN-γ production in *ex-vivo* blood at the time of diagnosis and during treatment follow-up.

The majority of dogs (53%) presented a strong *L. infantum* specific IFN-γ production at day 0 pre-treatment, and were mostly classified as being in moderate Leishvet stage IIa. CanL was thought to be associated with a strictly impaired specific T-cell mediated immunity and a humoral immunity overexpression (Pinelli et al., 1994). However, recent studies have shown that T-cell mediated immunity may also be detected in dogs with clinical disease (de Almeida Leal et al., 2014; Solano-Gallego et al., 2016b). In agreement to those studies, IFN-γ producer dogs commonly presented a mild to moderate clinical expression of the disease. The second group of naturally *L. infantum* infected sick dogs (47%) were found to be IFN-γ non-producers, and were mostly classified in higher clinical stages (IIb, III and IV), together with high specific antibody levels and blood parasitemia as previously described in other studies in dogs with severe clinical leishmaniosis (Boggiatto et al., 2010; Esch et al., 2013; Solano-Gallego et al., 2016b).

Several studies on leishmanial infection have attributed the development of antigen T-cell decreased functionality in CanL to T cell exhaustion which is characterized as antigen-specific effector T cell dysfunction with sustained expression of inhibitory receptors, including programmed cell death protein 1 (PD-1) and decreased effector cytokine production such as IFN-γ (Esch et al., 2013; Gigley et al., 2012). More recently, Chiku et al. found that the use of a PD-1 blocking agent in PBMC and spleen lymphocytes from dogs with leishmaniosis increases the production of nitric oxide and the parasite burden decreased (Chiku et al., 2016).

In another study, immunological characterization of an IgD^{hi} B cell subpopulation during CanL described a novel mechanism of IFN-γ T cell suppression through the PD-L1 and IL-10 pathways (Schaut et al., 2016). Schaut and collaborators observed an increment in IFN-γ production of T cells in response to specific *L. infantum* antigen after B cell PD-L1/IL-10 antibody blockade (Schaut et al., 2016). Considering all data previously reported, this specific mechanism might explain *L. infantum* antigen T cell unresponsiveness in our patients with moderate to severe disease.

It is important to highlight the significant increase of IFN-γ concentration after LSA stimulation in IFN-γ non producer dogs during long term treatment together with a decrease in *L. infantum* specific antibodies levels, blood parasitemia and clinical improvement while the IFN-γ producer group remained with similar IFN-γ concentrations during treatment follow-up as expected. Evaluation of IFN-γ production as a tool for evaluation of treatment monitoring has not been fully investigated in CanL. In agreement with the present study, the use of miltefosine and allopurinol during one year of treatment showed an increment in IFN-γ expression by RT-PCR (Manna et al., 2008b). Lower expression of IFN-γ was observed at the beginning of the treatment when compared to day 30 post-miltefosine together with a decreased parasite load in blood and lymph nodes, suggesting an association of this cytokine with parasite killing. However, three months after treatment, expression of IFN-γ was equal when compared with one month post treatment. At the final observation period (9–12 months), an increase of parasite burden in blood and lymph nodes together with a reduction of IFN-γ could be associated to failure in the Th1 response (Manna et al., 2008b). Evaluating the specific LSA *ex- vivo* IFN-γ response, we found that the majority of IFN-γ non producer patients did not present an immediate improvement in IFN-γ levels as revealed at day 30 of follow-up treatment. They needed a continued and prolonged treatment with allopurinol for more than 30 days to induce IFN-γ secretion. Furthermore, a considerable percentage of sick IFN-γ non-

producer dogs (50%) were not able to produce IFN-γ after one year of treatment albeit the evidence of reduction of antibodies and parasite load simultaneously with clinical improvement.

A recent study evaluated a heterologous vaccine (LBMPL) composed of *L. braziliensis* promastigote protein associated to monophosphoryl lipid A adjuvant in dogs with clinical leishmaniosis. LBMPL demonstrated induced secretion of IFN-γ from both CD4+ and CD8+ T lymphocytes associated with clinical improvement and decrease in parasite load (Roatt et al., 2017). These findings demonstrated a relationship between the detection of IFN-γ and the control of leishmaniosis and suggested that IFN-γ represents a good marker for predicting relapse of the disease in infected dogs and for determining the effectiveness of treatment. However, more conclusive results could be drawn if IL-4 is also measured concurrently with IFN-γ, based on the results of a recent study (Matralis et al., 2016).

Moreover, from the beginning of this study, IFN-γ producer dogs presented a good response to ConA mitogen, low antibody levels, blood parasitemia and mild clinical features that remained or improved along treatment indicating a good prognosis for these patients. In agreement, a study performed in cutaneous leishmaniosis in humans demonstrated that the capacity of *ex-vivo* production of IFN-γ by PBMC in non-healing patients was low after SLA stimulation and even in response to the powerful and non-specific mitogen phytohaemagglutinin (Moafi et al., 2017). Those data demonstrated that parasite specific IFN-γ secretion is a good CanL prognostic marker of mild to moderate clinical leishmaniosis. Supressed cell-mediated immunity in clinical CanL due to the incapacity of PBMCs to react to *Leishmania* antigen, is thought to explain the progressive outcome of this disease (Hosein et al., 2017).

In our present study, we reported the ability of a whole blood IFN-γ release assay to detect dogs with moderate or severe clinical leishmaniosis as previously reported in humans with VL due to *Leishmania donovani* (Singh et al., 2012; Singh and Sundar, 2014). In dogs, this kind of methodology was used to monitor IFN-γ production mainly in *Leishmania* vaccinated dogs (Costa-Pereira et al., 2015). To the best of our knowledge, the present study showed, for the first time, the use of WBA in a one year follow up after CanL treatment. In human VL, evaluating the stimulation effect of specific LSA in whole blood, has demonstrated advantages over the leishmanin skin test. The WBA demonstrated high specificity and good correlation with surrogate markers of exposures to *L. donovani*. The use of stimulated PBMC in confront with WBA has the limitation of intense modifying effects in T cell function causing pre-activation when cells were out of the physiological environment (Singh and Sundar, 2014). Additionally, the different laboratory conditions applied in these studies might have affected the production and secretion of IFN-γ by specific T cell subpopulations.

During VL, production of proinflammatory cytokines such as IFN-γ is generally made by both CD4+ and CD8+ T lymphocytes (Kaye and Aebischer, 2011). However, several studies has been demonstrated that T $CD4^+$ lymphocytes were the key and the source of IFN-γ production in *L. infantum* infected mice (Rosa et al., 2006) and in humans cutaneous leishmaniasis (Singh and Sundar, 2014). In naturally infected dogs, similar results were described by Esch et al. (2013) when antigen-responder IFN-γ CD4+ cells secreted higher amounts of IFN-γ than CD8+ T cells from PBMCs after LSA stimulation. Contrary, a recent study did not find any difference in the number of CD4 + IFN-γ+ and CD8+ IFN-γ+ lymphocytes for cytokine intracellular detection of IFN-γ in dogs with clinical leishmaniosis after LSA treatment (Matralis et al., 2016), although levels of intracellular and secreted cytokines may vary. Therefore, this assay is a promising tool to evaluate cellular immune responses in dogs in endemic areas at the time of diagnosis and during treatment follow-up.

Specific *L. infantum* antibodies showed an important decrease especially in the first 30 days of treatment, and then along the therapeutic schedule corresponding with clinical improvement in both groups of dogs. In agreement, previous reports described a reduction of antibodies in serum of moderate to severe clinically affected dogs (Solano-Gallego et al., 2016a) and also in dogs with a combined treatment regimen of aminosidine sulphate and meglumine antimoniate (Oliva et al., 1998). Some studies claimed that serology was not useful for treatment monitoring in the short term because in the first weeks of treatment the levels of specific anti-*Leishmania* antibodies do not correlate with the clinical course (Mateo et al., 2009). Recently, our group demonstrated that a serial dilution ELISA method is a useful tool for disease outcome evaluation since this technique allowed to evaluate the treatment efficacy as early as one month after treatment (Solano-Gallego et al., 2016a).

Accordingly with several investigations (Cavaliero et al., 1999; Pasa et al., 2005; Solano-Gallego et al., 2016a; Torres et al., 2011), our results showed that antibody levels continued to decrease through the treatment period but less pronouncedly. As demonstrated here and in agreement with Rodriguez et al. (2006), the minority of dogs became seronegative during the first year of treatment, however all sick animals under study reached lower levels of specific antibodies when compared to the time of diagnosis. It is well known that treatment does not achieve a parasitological cure in the majority of dogs although most treated dogs demonstrate a clinical cure (Hosein et al., 2017; Manna et al., 2008a). Therefore, persistent antigenic stimulation is likely to occur in some dogs but a progressive decline of antibodies usually follows a decrease in parasite loads in these dogs. It is important to remark the high variability in the levels of specific *L. infantum* antibodies between dogs in similar clinical stages, as described previously in other studies (Solano-Gallego et al., 2016a; Torres et al., 2011). Importantly, both groups (IFN-γ producers and IFN-γ non-producers) significantly decreased antibody levels together with clinical improvement along treatment although IFN-γ non-producer dogs remained at the end of treatment follow-up with higher levels of antibodies when compared with IFN-γ producers.

Consistent with previous studies, we detected a significant decline in the blood parasite load during the first 30 days of treatment and a maintenance of reduced parasitemia through all the treatment period (Baneth and Shaw, 2002; Manna et al., 2008a; Martinez et al., 2011; Solano-Gallego et al., 2016a). However, levels of parasitemia were detected along treatment follow-up even though at low levels in comparison with other studies (Manna et al., 2008a; Manna et al., 2008c). *Leishmania infantum*-infected dogs, whose blood, lymph nodes and skin samples were screened by RT-PCR showed that during a long period of treatment, a progressive decrease of parasite load was associated with clinical recovery, even though a marked parasite load was still present in lymph nodes in 50% of dogs after 24 months of treatment (Manna et al., 2008c). Additionally, in our study, presence of *L. infantum* DNA was detected in some dogs after negative RT-PCR results were obtained in preceding follow-up samples. A possible explanation could be an intermittent parasitemia, described by other authors when RT-PCR was used to correlate the *Leishmania* DNA load and cytokine expression levels (IFN-γ and IL-4) in tissue samples from CanL dogs (Manna et al., 2008a). Intermittent parasitemia was associated with a clinical relapse based on increased antibody levels, clinical signs and/or clinicopathological abnormalities. Therefore, blood RT-PCR during treatment monitoring should be accompanied by a full physical examination, quantitative serology and routine laboratory tests. Importantly, in our study the majority of treated dogs achieved a clinical cure while parasitological load did not completely vanish as previously reported (Manna et al., 2008c).

In accordance with the clinical improvement, abnormal clinicopathological findings at the time of diagnosis were normalizing during the treatment period as previously described (Cavaliero et al., 1999). No apparent adverse effects were associated with treatment as assessed by clinical, hematologic, and blood chemistry testing. Gamma-globulins substantially decreased and albumin increased along the follow-up, as reported in other studies (Pierantozzi et al., 2013). It is recognized that increased gamma-globulins concentration is correlated with a high parasitic load, and achieving normal gamma-globulins

values is a good marker of treatment effectiveness. Proteinuria in CanL is a common finding, related to a renal glomerular injury. In our study, both groups of dogs had proteinuria and through the treatment, IFN-γ non-producer dogs reduced the urine protein creatinine ratio values, as expected and reported in a previous study (Pierantozzi et al., 2013).

5. Conclusions

Data presented here, confirms that sick dogs lacking *L. infantum* specific IFN-γ production in stimulated whole blood produce a strong humoral response, have a high blood parasitemia and severe clinical disease. Our results demonstrated that IFN-γ plays a key role in the forecasting the prognosis of *L. infantum* infection since increasing IFN-γ concentration observed during long-term anti-*Leishmania* treatment in dogs initially lacking IFN-γ production were associated to clinical improvement. In addition, a significant reduction of antibody levels and improvement in the levels of the majority of clinicopathological parameters were observed during monitoring in both groups of dogs studied (IFN-γ producers and IFN-γ non-producers). However, blood parasitemia was a more limited value as marker for diagnosis or treatment monitoring purposes in both groups studied.

Conflicts of interests

The authors declare that they have no competing interests.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vetpar.2017.10.018.

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