Humoral and T-cell mediated immunity against *Phlebotomus perniciosus* salivary proteins in dogs from a leishmaniosis endemic area

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Núria Balsells Aguilar

Finals d´anys 80.

I el veterinari ens va dir:

"Té leishmaniosi.

És una malaltia del que se´n sap poc.

Encara cal molta investigació…"

Agraïments

Primer de tot, moltes gràcies a la Laia Solano Gallego, per donar-me la gran oportunitat de fer aquest treball. Després de molts anys dedicats a la clínica de petits animals ha estat un privilegi poder-me endinsar en el coneixement d´una malaltia, que per sort o desgràcia, he pogut conèixer massa bé al llarg del temps. Moltes gràcies a la Marta Baxarias i a la Lourdes Alarcón, per el seu suport i paciència. Les hores es fan curtes al vostre costat! Gràcies també a la Magdalena Alcover per les aportacions sobre flebotoms i també durant el necessari treball de camp, juntament amb l´Alejandra Álvarez.

Sens dubte, res no hauria estat possible sense el suport del Xavi. M´has animat des del primer moment, aguantat les meves angoixes i mal humors, has fet de pare i mare, cuiner, taxista i mil i una coses més per tal que pogués tirar endavant aquest projecte. T´estimo.

Gràcies, mare, per els mil i un missatges de suport al llarg de tots aquests mesos de confinament. Encara que ens hem vist poc, per salvaguardar la teva salut, has estat sempre ben present. I el record del pare no ha faltat mai.

Dedicat al Pol, Ferran, Anna, Adam i Lydia. El proper TFM l´heu de fer vosaltres.

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Abbreviations

Abbreviation	Meaning
CanL	Canine Leishmaniosis
CL	Cutaneous Leishmaniosis
ConA	Concanavalin A
ELISA	Enzyme-Linked Immunosorbent Assay
IFN-γ	Interferon Gamma
Ig	Immunoglobulin
IL	Interleukin
IQR	Interquartile Range
LSA	*Leishmania infantum S*oluble Antigen
MCL	Muco-Cutaneous Leishmaniosis
NO	Nitric Oxide
OD	Optical Density
SD	Standard Deviation
SE	Standard Error
SGH	Salivary Gland Homogenate
SOD	Standardized Optical density (ELISA units)
Th1	Type 1 T helper cell
Th2	Type 2 T helper cell
TNF-α	Tumor Necrosis Factor alpha
UK	United Kingdom
USA	United States of America
VL	Visceral Leishmaniosis
WHO	World Health Organization

Abstract

Background: Leishmaniosis is a neglected zoonosis transmitted by sand fly bites and the dog is the main reservoir of *Leishmania infantum* infection. Sand fly exposure could elicit T-cell mediated immunity against sand fly saliva proteins, a mechanism that could be protective against a negative outcome of the disease and could explain the natural resistance of Ibizan hounds toward leishmaniosis.

Objective and methods: This study aimed to explore the humoral and T-cell mediated immunity against *P. perniciosus* salivary proteins in Ibizan hounds (n=51) and dogs of other breeds (n=34) living in the island of Mallorca (Spain), an endemic area for canine leishmaniosis, and to correlate anti-saliva immune responses with clinical, immunological and parasitological parameters. Anti-sand fly saliva IgG was examined using two methods: *P. perniciosus* whole salivary gland homogenate (SGH) ELISA and recombinant protein rSP03B ELISA. Furthermore, a whole blood assay with *L. infantum* soluble antigen, salivary gland extract and recombinant protein rSP03B were performed to measure IFN-γ production in order to assess T-cell mediated immunity and establish a correlation with anti-sand fly saliva IgG and clinical, immunological and parasitological parameters of canine leishmaniosis.

Results: Significant agreements were found between anti-sand fly saliva IgG levels for proteins SGH and rSP03B, IFN-γ production related to SGH and rSP03B salivary proteins and IFN-γ production related to *L. infantum* and *Leishmania*-specific antibody levels. Low concentrations of IFN-γ SGH and IFN-γ rSP03B are found in less than 20 % of dogs, in contrast with higher levels of IFN-γ LSA and IgG anti salivary gland homogenate and recombinant protein rSP03B. Higher levels of IFN-γ LSA also seem to be associated to age and *Leishmania*- specific antibodies

Conclusions: A relation was established between *P. perniciosus* saliva and immune responses against *Leishmania* infection. Even if it was not always possible to prove in this study that Ibizan hounds and other breeds had different levels of humoral and T-cell mediated immunity against salivary gland proteins, a connection was found between IFN-γ SGH, IFN-γ rSP03B and all the other parameters evaluated, concluding

that dogs living in endemic areas for leishmaniosis present cellular and humoral immunity against *P. perniciosus* salivary proteins.

Keywords

Canine leishmaniosis, *Leishmania infantum*, *Phlebotomus perniciosus* salivary proteins, Ibizan hounds, anti-saliva antibodies, specific *P. perniciosus* saliva T-cell mediated immunity.

Background

Introduction

Leishmanioses are a group of neglected zoonosis caused by protozoans of the genus *Leishmania* (class Kinetoplasta, family Tripanosomatidae), endemic in large geographic areas with a worldwide distribution. There are around 53 known species of *Leishmania*, 31 of them are parasites to mammals and 20 of them are known to cause disease to humans (Akhoundi et al., 2016).

In humans, leishmaniosis has several disease presentations linked to different species of *Leishmania*, observing variable endemic rates according to its geographical location (Aronson et al., 2017). Cutaneous leishmaniosis (CL) is the most frequent form of the disease, causing painless and chronic skin lesions often occurring at sites of sand fly bites, and usually healing spontaneously as cell mediated immunity develops. Mucocutaneous leishmaniosis (MCL) is a form of the disease causing destructive and disfiguring lesions of the naso-oropharyngeal and laryngeal mucosa. Visceral leishmaniosis (VL), also known as kala-azar, is a life-threatening condition when treatment is not provided, often seen as an opportunistic infection in immunosuppressed patients (Aronson et al., 2017). According to WHO, in 2018, 92 countries were considered endemic for human leishmaniosis, with 1 billion people at risk of infection and an estimation of 600.000-1 million new cases diagnosed every year (WHO 2018).

Leishmaniosis is usually transmitted to mammal hosts by infected female phlebotomine sand flies, mainly of the genera *Phlebotomus* in the Old World and

Lutzomyia in the New World (Ribeiro et al., 2018), acting as vectors of these parasites, although some other species of sand flies are also vectors of *Leishmania*.

From 800 species of *Phlebotomus* known worldwide, less than 100 of them are suspected or proven *Leishmania* vectors (Lestinova et al., 2017). Several *Phlebotomus* species are identified across the Mediterranean basin, being *Phlebotomus perniciosus* the predominant species in Spain (Alten et al., 2016; Gálvez et al., 2020).

Infection occurs when an infected sand fly inoculates metacyclic promastigotes to the host during blood feeding, together with saliva. Sand fly saliva is composed of different molecules with pharmacological and immunomodulatory properties (Lestinova et al., 2017). Clinical outcome of the infection depends on the virulence of the parasite strain but also on the host´s genetic background and immune status (Lestinova et al., 2017).

The range of hosts for leishmaniosis involve around 70 species of mammals, including xenarthrans, hyraxes, marsupials, chiropterans, lagomorphs, procyonids, felids, perissodactyla and primates, acting as reservoirs of the parasite in the nature, although their role on the epidemiology of this disease is not fully understood (Ribeiro et al., 2018). Natural infection is more common in rodents and canids (Ribeiro et al., 2018), being the domestic dog the main reservoir for *L. infantum* infection (Travi et al., 2018). It has been estimated that at least 2.5 million dogs are infected in south Western Europe alone (Baneth et al., 2008). In the Old World, *L. infantum* is the main parasite specie affecting dogs, although *L. major* and *L. tropica* also have been isolated from them (Baneth et al., 2017) whilst in the Americas the *Leishmania braziliensis* complex is the predominant (Brilhante et al., 2019).

Canine leishmaniosis due to *L. infantum* distribution is almost worldwide; the disease is present in regions of Southern Europe, Africa, Asia, South and Central America and also reported in the United States of America (Solano-Gallego et al., 2011), with high prevalence of leishmaniosis seen in the Mediterranean Basin (Velez et al., 2019) and South America (Solano-Gallego et al., 2008). An increased prevalence of this infection in non-endemic territories is expected in the future, where climate change would promote a rise of vector and parasite presence, together with every time more frequent imported cases due to travelling and relocation (Maia & Cardoso, 2015).

Equally, to humans, dog susceptibility and affectation depends on host's immune response, leading to a wide range of clinical presentations. Some individuals are subclinical, and infection is unapparent whilst others may show a variety of clinical signs and/or clinicopathological abnormalities, from cutaneous lesions to severe systemic disease (Solano-Gallego et al., 2011). Interactions between host and parasite are complex so clinical manifestations and progress of disease depends on that (de Vasconcelos et al., 2019).

Some dog breeds are reported to be more susceptible to systemic disease, such as Boxer, Cocker Spaniel, Rottweiler and German shepherd (Solano-Gallego et al., 2011). On the other hand, Ibizan hounds seem to be more resistant to *Leishmania* infection due to a significant parasite specific T cell immune cellular response (Solano-Gallego et al., 2000), showing higher prevalence of papular dermatitis instead of systemic disease, compared to other dog breeds (Burnham et al., 2020).

Sand fly saliva overview

Although several forms of non-vectorial transmission have been reported as well in dogs, such as transplacental, venereal, through blood transfusion (Maia & Cardoso, 2015); Solano-Gallego et al., 2011). Dog to dog transmission through bites and wounds has not been proved (Solano-Gallego et al., 2011). Leishmaniosis transmission is mainly linked to sand fly feeding success, with injection of saliva to the host during blood meals together with parasites (Lestinova et al., 2017). A blood meal is a process that inflicts some damage to the host´s skin, being this damage usually counteracted by host´s mechanisms of hemostasis, inflammation and immunity (Abdeladhim et al., 2014). Sand fly saliva has a variety of pharmacological substances which interfere with the host hemostasis and immune response (Rohoušová & Volf, 2006). Furthermore, sand fly saliva promotes infection establishment thanks to its antihemostatic, anti-inflammatory and immunomodulatory properties that help to complete the blood meal (Lestinova et al., 2017).Sand fly saliva is also chemotactic for different immune cells and able to modify inflammatory processes at feeding site (Lestinova et al., 2017). Many cell types would interact with the parasites, such as dendritic cells, neutrophils and macrophages (Hosein et al., 2017).

Saliva composition differs among different species and populations of sand flies from different geographical areas (Lestinova et al., 2017), physiological stage of the adults and sex (Rohoušová & Volf, 2006). Interestingly, different concentrations of salivary proteins between males and females are encountered, with hematophagous females showing larger numbers of salivary proteins than nonhematophagous males (Lestinova et al., 2017).

Salivary proteins are grouped in several families, with different biological functions and antigenic properties. Host´s hemostasis is counteracted by proteins such as apyrase, acting on platelet aggregation and maxadilan, a compound isolated from *L. longipalpis*; Odorant binding proteins, such as SP15-like proteins. Other salivary proteins can remove biogenic amines (serotonin, histamine, catecholamines) from the feeding site. Blocking amines leads to vasodilation, platelet deactivation and decreased vascular permeability. There are three groups of amine binding proteins found in saliva: lipocalins, D-7 proteins and YRPs (Yellow Related Proteins) (Lestinova et al., 2017). However, amine binding ability is only described for YRPs (Sumova et al., 2019), suggesting that all YRPs could bind to various biogenic amines with different affinities. Other substances found in sand fly saliva include hyaluronidases and endonucleases, with degrading functions over the extracellular matrix of host´s skin, helping saliva spreading over other active compounds (Lestinova et al., 2017).

Sand fly saliva can modulate the host immune response at innate and acquired level, with a wide range of effects observed that could sometimes differ in saliva from *Phlebotomus* or *Lutzomyia* (Rohoušová & Volf, 2006). *Phlebotomus* saliva produces some inhibitory effect on lymphocyte proliferation, suppression on early production of interleukin 2 (IL-2), interleukin 4 (IL-4) and interferon gamma (IFN-γ), increased interleukin 6 (IL-6) production and induction of positive macrophage chemotaxis. Nitric oxide (NO) synthesis in macrophages would be altered due to down regulation of NO synthase (Rohoušová & Volf, 2006).

Host immune response

Many factors act over the host capacity to be resistant or susceptible to disease, such as age, sex, breed, immunosuppression, presence of concomitant disease or ongoing coinfections, nutritional status and proportion of time spent outdoors (Hosein et al., 2017).

Genetic components related to breed also play a role on the outcome of the disease, as many loci are accountable for disease progression (Quilez et al., 2012). Resistance to disease, as usually seen in Ibizan hounds, is associated with development of strong Th1 response, with production of IFN-γ, Tumor Necrosis Factor alpha (TNF-α) and IL-2, with a mixed Th1/Th2 response observed in active disease (Hosein et al., 2017). Immune responses to the parasite are organ specific and cytokine profiles are variable in different body tissues (Hosein et al., 2017). The main effector mechanism to kill *Leishmania* parasites is macrophage activation by IFN-γ and TNF-α (Hosein et al., 2017) enhancing the production of NO and other toxic intermediates by macrophages, resulting in destruction of parasites (Lestinova et al., 2017). On the other hand, susceptible hosts tend to have a stronger Th2 response with enhanced production of type 2 cytokines, which leads to selective inactivation of some macrophage functions and increased humoral response unable to control the infection.

In endemic areas of canine leishmaniosis, there is evidence that hosts repeatedly exposed to sand fly bites are also capable to develop anti-saliva antibodies, mostly IgG, as well as a cellular response to sand fly saliva (Kostalova et al., 2015; Matralis et al., 2016; Lestinova et al., 2017). Seasonal exposure to sand flies is related to the period of activity and abundance of this vectors and could lead to antibody fluctuations along the time (Kostalova et al., 2015; Vlkova et al., 2011), also, older dogs have experienced more sand fly seasons, so they are expected to have been more exposed to sand flies and develop higher immunity levels than younger dogs. (Kostalova et al., 2015; Burnham et al., 2020). Ibizan hounds could be more exposed to sand fly bites due to the vulnerability of their ear pinnae: large, pointed upright and hairless, easy to reach by sand flies. According to previous studies, Ibizan hounds show predominantly cellular parasite-specific immune responses, with greater IFN-γ production and higher levels of anti-P-perniciosus saliva antibodies (Burnham et al., 2020), and that could be related to their resistance to leishmaniosis, usually showing mild forms of the disease with

more prevalence of papular dermatitis (Ordeix et al.,2005; Burnham et al., 2020). Despite of these facts, little studies have been performed to investigate the relationship between humoral and cellular immune responses in dogs.

Hypothesis and objectives

Sand fly saliva compounds can elicit specific immune responses that have a significant role in *Leishmania* establishment in the dog and disease outcome. Characterizing anti-saliva immune responses in dogs living in well-defined leishmaniosis endemic areas would provide valuable insights regarding their effect on parasite transmission, modulation of parasite specific immune responses, parasite establishment and infection outcome in dogs. To the best knowledge of the authors, canine cellular immune response against *P. perniciosus* salivary proteins has not been investigated in dogs living in endemic areas for leishmaniosis. The aim of this study was to explore the humoral and T-cell mediated immunity against *P. perniciosus* salivary proteins in Ibizan hounds and dogs of other breeds living in the island of Mallorca (Spain), and to correlate anti-saliva immune responses with clinical and immunological parameters.

The study hypothesizes that Ibizan hounds will have higher levels of anti-*P. perniciosus* saliva IgG antibodies than dogs of other breeds due to increased exposure to sand fly bites. Furthermore, a positive correlation is expected between anti-*P. perniciosus* saliva IgG antibodies and T-cell mediated immunity, as determined by IFN-γ production.

The research specific objectives were as follows:

- To determine and compare levels of anti-*P. perniciosus* saliva antibodies in Ibizan hounds and other dogs of other breeds living in Mallorca. Anti. *P. perniciosus* saliva antibodies were evaluated by two different methods: 1) ELISA using the antigen *P. perniciosus* SGH, 2) ELISA based on the yellow related recombinant protein of *P. perniciosus*, rSP03B.
- To determine and compare levels of *P. perniciosus* specific IFN-γ production in Ibizan hounds and dogs of other breeds and correlate levels of anti-*P.*

perniciosus saliva antibodies and T-cell mediated immunity with clinical and immunological parameters.

Materials & Methods

Dogs enrolled to this study were from Mallorca, the largest Balearic Islands, an archipelago in the western Mediterranean Sea. Balearic Islands are an endemic area for canine leishmaniosis. According to recent studies, seroprevalence for canine leishmaniosis was around 10 % in Spain, but in the Balearics, seroprevalence showed to be as high as 57% (Gálvez et al., 2020). Due to latitude and mean yearly temperature, presence of active *Phlebotomus* can be detected in warm periods of the year, acting as transmission vectors of leishmaniosis for the dogs inhabiting this area (Izri et al., 2006).

Dogs

86 owned dogs were enrolled in September 2019 from Mallorca (Spain). 51 dogs were Ibizan hounds and 34 dogs from other breeds. Dogs were living in the same area and had the same environmental conditions, such as staying outdoors and by that, be exposed to sand fly bites. Basic clinical characteristics were recorded, including breed, age (from 4 months to 12 yrs. old), sex (male *n=24*, female *n=60*) and clinical status. In this study, dogs were categorized as adults when their age was over 18 months old. Blood samples were taken to obtain serum and propagate primary whole blood cultures and for determination of anti-*P. perniciosus* IgG. Clinical, parasitological and *L. infantum* specific immunological parameters were also evaluated. A signed consent form was obtained from tutors of the dogs enrolled.

Whole blood assay

After taking the blood samples from both group of dogs, heparinized whole blood was diluted to a ratio of 1:10 with Rosewell Park Memorial Institute (RPMI) 1640 medium with stable glutamine and 25 mM hepes (Biowest®, USA) supplemented with penicillin and streptomycin (Life Technologies TM, USA) and 10% Fetal Bovine Serum Premium South America Origin (Biowest®, USA). 50 µl of heparinized blood was mixed with 450

µl of complete medium in each well. Duplicates were made for each condition, except for recombinant antigen due to its limited availability.

Five different conditions were established to stimulate the whole blood:

(i) Medium alone.

(ii) Medium with *L. infantum* soluble antigen (LSA) at a concentration of 10 µg/ml provided by Dr. Cristina Riera *(Facultat de Farmacia, Universitat de Barcelona).*

(iii) Medium with mitogen concanavalin A (ConA) (100 mg Medicago®, Sweden) at a concentration of 10 µg/ml.

(iv) Medium with *P. perniciosus* salivary gland homogenate (SGH), provided by Professor Petr Volf (*Charles University of Prague*) at a concentration of 1 salivary gland/ml.

(v) Medium with *P. perniciosus rSPO3B* recombinant protein, provided by Professor Petr Volf (*Charles University of Prague*) at a concentration of 20 µg/ml.

The plates were incubated in 48-well flat bottom plastic culture plates 30048 (SPL Life Sciences Co., Ltd., Korea) for 5 days at 37 °C in 5% of CO_2 air. Following 5 days of incubation, blood was centrifuged at 323 g, for 10 minutes (Heraeus Labofuge 400R) and the supernatant was collected and stored at -80 °C until used to perform an IFN-γ determination**.**

Sandwich ELISAs for the determination of IFN-γ concentration

After a whole blood assay, an optimized ELISA was used to measure IFN-γ production. Cytokine analysis of IFN-γ was performed according to the manufacturer's instructions (DuoSet® ELISA by Development System R&D™, UK) using 96 well cell flat bottom plates (ref. 3590, Costar® Corning, USA). Slight modifications were done for the IFN-γ ELISA. The standard curve for IFN-γ started with 2000 pg/ml and two-fold dilutions were made until 15,65 pg/ml concentration. Supernatants treated with ConA were diluted 1:1 with reagent diluents. Duplicates of all supernatants studied were performed in all ELISAs. Optical density was measured with an ELISA reader (MB-580, Shenzhen Heales Technology Development Co., Ltd, China) at a wavelength of 450 nm.

The standard curve for IFN-γ was calculated using a computer generated four parameter logistic curve-fit with program MyAssays (http://www.myassays.com/). Plates were repeated when R^2 value of the standard curve was below 0.98.

Dogs were classified as IFN-γ producers for *L. infantum* specific IFN-γ when concentration was over 62.5 pg/ml after subtracting medium alone. This value was taken as a reference for the cut off according to previous study (Solano-Gallego et al., 2016). Also, dogs were classified as IFN-γ producers for SGH and rSP03B when IFN-γ concentration was detectable after subtracting medium alone and classified as IFN-γ non-producers when concentrations were not at detectable levels.

Indirect enzyme-linked immunoabsorbent assay (ELISA) for the determination of L. infantum

All dogs had a quantitative in-house serology ELISA to measure specific antibodies against *L. infantum*, performed by means of a serial dilution. 96-well ELISA plates (reference 3590, Costar® Corning, USA) were coated with 100 µl of *L. infantum* suspension at a concentration of 20 µg/ml in carbonate buffer 0.05M, pH 9.6 and then incubated at 4 °C overnight. 100 µl of canine sera was added at a 1/800 dilution in phosphate saline buffer (PBS, pH 7.4) (Sigma), with 0.05 % Tween 20 (PBTS) and 1% low fat dry milk (PBTSL) and incubated at 37°C for 60 minutes. High positive and negative controls, as well as positive calibrator and conjugate controls were added in each plate. After washing the plates three times with PBST, 100 µl of Protein A peroxidase (Thermo scientific, reference 32400) conjugate was added using a 0.16 ng/ml concentration and incubated at 37°C for 60 minutes. The solution was developed after another series of the above washings described, using 200 µl of substrate solution 0.4 mg/ml OPD, 0.4 mg/ml urea hydrogen peroxide, and 0.05 M phosphate-citrate, pH 5.0 (SIGMA FAST™ OPD tablet, Sigma) and then incubated 5-20 minutes at room temperature. Reaction was stopped adding 50 µl of H_2SO_4 3M. Reading was done with an ELISA reader (MB-580, Shenzhen Heales Technology Development Co., Ltd, China) at 492 nm.

Results were quantified using ELISA units related to the positive calibrator, given 100 EU to an optical density approximately of 1. Cut off was established at 35 EU (median +

4 SD of 80 dogs of a non-endemic area). To calculate results, EU obtained in problem sera were divided by calibrator EU and then multiplied by 100. Results ≥ 300 EU were considered high positive, medium positive ≤ 150 and < 300, low positive <150 and >35 and negative <35 EU. According to these values, dogs were initially classified as negative (n=55), weak positive (n=27) and medium positive (n=3). However, because of the low number of dogs classified as medium positives, the analysis was made considered all dogs positive and negative.

Indirect enzyme-linked immunoabsorbent assay (ELISA) for the determination of anti-P. perniciosus IgG

A whole salivary gland homogenate of *P. perniciosus* females as well as recombinant salivary proteins (rSP03B) were used for this purpose, provided by Professor Petr Volf at Charles University in Prague, who has a colony of *P. perniciosus* and *P. Perniciosus* recombinant salivary proteins.

Sera samples were analysed by indirect enzyme linked immunoabsorbent assay (ELISA) measuring anti-*P. perniciosus* IgG by using *P. perniciosus* salivary gland homogenate (SGH) as previously described (Burnham et al., 2020) and recombinant protein rSP03B with slight modifications (Burnham et al., 2020). After reconstitution of lyophilized rSP03B and *P. perniciosus* SGH with dH_2O, 96-well ELISA plates (reference 3590, Costar® Corning, USA) were coated with *P. perniciosus* salivary gland homogenate (SGH) (0.2 salivary gland per well) or recombinant protein rSP03B (0.1 µg per well) diluted in carbonate-bicarbonate buffer (pH9, 100 µl per well) and incubated overnight at 4˚C. The plates were washed twice with PBS + 0.05% Tween 20 (PBTS) (100 µl per well) and blocked with 6% low fat dry milk diluted in PBTS (100 µl per well) for 60 minutes at 37°C. After that, plates were washed three times with PBS-Tw (100 µl per well).

Serum samples were diluted 1:100 (for rSP03B) and 1:200 (for SGH) in 2% low fat dry milk and added to the plates. Each sample was made at least in duplicate, incubated for 90 min at 37°C and washed five times with PBTS (100 µl per well). Plates were incubated again at 37 °C for 45 min after adding a conjugate (HRP; peroxidase-

conjugated goat anti-dog IgG antibody (Bethyl Laboratories A40-123P, diluted 1:9000 in PBTS (100 µl per well) and washed five times with PBTS.

The ELISA was developed using a substrate solution (200 µl per well) made with 0.4 mg/ml OPD, 0.4 mg/ml urea hydrogen peroxide, and 0.05 M phosphate-citrate, pH 5.0 (SIGMA FAST^{TM} OPD tablet).

The plates were incubated in the dark at room temperature and the reaction was stopped after 5-20 min with 50 µl 10% $H_2SO_4$5 N and absorbance (OD value) was measured at 492nm using a ELISA reader (MB-580, Shenzhen Heales Technology Development Co., Ltd, China).Each serum was tested in duplicate.

Cut off value for SGH was performed in accordance with a previous study (Burnham et al., 2020). Cut off value for rSP03B was calculated using 26 control residual samples obtained from Royal Veterinary College (London, UK) from a random set of dogs presenting at the Queen Mother Veterinary Teaching Hospital (Burnham et al., 2020), together with 10 control residual samples from experimental healthy Beagles from Barcelona. It was assumed that these dogs were unexposed to sand flies. High positive and negative controls, as well as positive calibrator and conjugate controls were added in each plate. Mean value + 3 SD were calculated and a final value of 51 ELISA units was used as a cut off.

Statistical analysis

Data analysis was performed with a combination of R software (http:// cran.r-project.org/) and Quickcalcs from Graphpad (www.graphpad.com/quickcalcs/). Diagnostic performance was calculated based on degree of agreement between pairs of tests, using a McNemar´s test and Cohen´s kappa. Correlations between results of the performed tests were estimated using Spearman rho correlation and linear regression, after transforming ELISA units to logarithmic values to normalize residuals and stabilize the variance. Mann-Whitney-Wilcoxon test was used to examine intra group differences in median values of ELISA units between tests performed and clinical parameters. Proportions of intra group positive results were compared using Fisher's

exact test. Results were expressed as medians (interquartile range, IQR) and p-values < 0.05 were considered statistically significant.

Results

Description of data of Ibizan Hounds and group of dogs of other breeds

A total of 85 dogs were enrolled for this study, 51 of them were Ibizan Hounds and the rest (n=34) were dogs of other breeds, including setter (n=3), Mallorca shepherd dog (n=3), Border collie (n=2), ratter (n=2), greyhound (n=2), German shepherd dog (n=1), carline (n=1). Seventeen crossbreed dogs were included, mainly derived from crosses between hunting or working dogs and the previous described breeds. Data about breed was missing in three dogs.

The median age of the entire sample of dogs was 24 months (range 6-168 months, one dog without age recorded), with 50/85 (59.5%) of them above 18 months of age and qualified as adults in this study. When comparing both groups, Ibizan Hounds had a lower median age and lower proportion of adult dogs (median: 18 months; 23/50 or 46% adults) compared to the group of other breeds (median: 36 months; 27/34 or 79.41% adults) (Mann-Whitney: p= 0.0253 for comparison of median ages; Fisher´s exact test: p= 0.0030 for proportion of adult dogs).

Regards to sex, the proportion of male and female was equal within the group of other breeds (17/34 or 50% - equal for each sex) in contrast with Ibizan Hounds, with a greater proportion of females (43/50 or 86%), finding a statistical significant difference between the two groups (Fisher´s exact test: p= 0.0005).

Comparison of agreement of tests performed

Pair wise analysis to compare agreement between the two salivary antibody tests (SGH ELISA, rSP03B ELISA) and the three IFN-γ tests (IFN-γ LSA, IFN-γ SGH, IFN-γ rSP03B) were performed, and the results are listed in Table 1. As pictured, a 75.79% of agreement between both salivary antibody tests, SGH and rSP03B, was observed, with

a kappa indicating a moderate degree of agreement between them. However, McNemar exact test determined a statistically significant difference in the proportion of positive responses from SGH ELISA and rSP03B ELISA (p=0.0088).

Table1. Comparison of agreement between the *P. Perniciosus* salivary antibody tests and IFN-γ ELISA antibody tests performed.

	Percent agreement	McNemar exact test p-value	Kappa± SE	Kappa interpretation †
SGH ELISA vs. rSP03B ELISA	75.29%	0.0088	0.515±0.088	Moderate agreement
SGH ELISA vs. IFN-γ LSA	57.65%	0.0668	0.045±0.104	Slight agreement
SGH ELISA vs. IFN-γ SGH	41.18%	0.0162	-0.126±0.100	No agreement
SGH ELISA vs. IFN-γ rSP03B	35.29%	0.0005	-0.185±0,092	No agreement
rSP03B ELISA vs. IFN-γ LSA	47.06%	0.0003	-0.016±0.090	No agreement
rSP03B ELISA vs. IFN-γ SGH	49.41%	0.5419	-0.029±0.108	No agreement
rSP03B ELISA vs. IFN-γ rSP03B	45.88%	0.0553	-0.120±0.100	No agreement
IFN-γ LSA vs. IFN-γ SGH	41.8%	<0.0001	0.068±0.085	No agreement
IFN-γ LSA vs. IFN-γ rSP03B	44.71%	<0.0001	0.085±0.066	Slight agreement
IFN-γ SGH vs. IFN-γ rSP03B	65.88%	0.1374	0.256±0.105	Fair agreement
***Leishmania* ELISA *vs.* SGH ELISA**	62.35%	0.0002	0.294±0.088	Fair agreement
***Leishmania* ELISA vs. rSP03B ELISA**	47.06%	0.0001	0.061±0.081	Slight agreement
***Leishmania* ELISA vs. IFN-γ LSA**	50.59%	0.0001	0.140±0.073	Slight agreement
***Leishmania* ELISA vs. IFN-γ SGH**	50.59%	0.6434	-0.050±0.107	No agreement
***Leishmania* ELISA vs. IFN-γ rSP03B**	61.18%	0.4862	0.117±0.110	Slight agreement

† the interpretation of kappa value is shown in the final column according the following scale: ≤0, no agreement; 0.00-0.20, slight; 0.21-0.40, fair; 0.41-0.60, moderate; 0.61-0.80, substantial; 0.81-1, almost perfect.

Abbreviations: ELISA, enzyme-linked immunosorbent assay; IFN-γ, interferon gamma; LSA, *Leishmania* soluble antigen; SE, standard error; SGH, salivary gland homogenate.

Slight agreement was observed (57.65%) between SGH ELISA and IFN-γ LSA. Low percentage of agreement (41.8%) was observed between IFN-γ LSA and IFN-γ SGH, and a similar result between IFN-γ LSA and IFN-γ rSP03B (44.71%), with kappa indicating slight or no agreement between them. In both cases, McNemar exact test p-value was

<0.0001, indicating statistically significant difference in the proportion of positives for both combination of tests. Fair agreement was observed between IFN-γ SGH and IFN-γ rSP03B (65.88%) with no statistically significant difference in the proportion of positive responses (p=0.1374). Slight or no agreement was observed between test results when comparing combinations of SGH and rSP03B with either IFN-γ.

Fair amount of agreement according to kappa values was seen when comparing *Leishmania* ELISA and SGH ELISA, with a 62.35%. Also, slight agreement was observed as well between *Leishmania* ELISA and rSP03B ELISA (47.06%), IFN-γ LSA (50.59%) and IFN-γ rSP03B (61.18%).

Spearman rho correlation and linear regression

After converting ELISA units into their logarithmic values, a Spearman rho correlation was performed to identify possible relationships between tests performed and clinical parameters. Table 2 shows the relationship between SGH and rSP03B ELISA units and quantitative clinical parameters. Significant Spearman´s correlation was observed

Table 2. Relationship between log salivary ELISA units and other quantitative clinical parameters using Spearman rho correlation and linear regression.

	Log salivary ELISA units			
Clinical Parameters	**SGH**		**rSP03B**	
	r_2	**P-value**	**r_2**	**P-value**
Log *Leishmania* specific antibodies	0.4077	0.0001***	0.2742	0.0011**
Log IFN-γ LSA	0.1654	0.1302	0.0938	0.3928
Log IFN-γ SGH	-0.0418	0.7050	0.0679	0.5363
Log IFN-γ rSP03B	-0.2276	0.0361*	-0.1515	0.1661
Age (months)	-0.0326	0.7682	-0.9398	0.3951
Log rSP03B	0.7024	<0.0001***		

Log_{10} transformations were performed for each variable involving ELISA units (salivary antigens, *Leishmania* antigen, and IFN-γ antigen).

Significant p-values are designated with an asterisk (*p<0.05, **p<0.01, ***p<0.001).

Abbreviations: ELISA, enzyme-linked immunosorbent assay; IFN-γ, interferon gamma; LSA, *Leishmania* soluble antigen; SE, standard error; SGH, salivary gland homogenate.

between log ELISA units for *Leishmania* specific antibodies for both SGH (p=0.0001) and rSP03B (p=0.0011). Similarly, some correlation was observed between log IFN-γ rSP03B concentration values and SGH ELISA units (p=0.0361). Finally, a strong correlation was found between log ELISA units for rSP03B and SGH.

When analysing correlations between log IFN-γ concentration values and other quantitative clinical parameters (Table 3), significant correlations were observed between log Leishmania specific antibodies and log IFN-γ LSA (p=0.0121) and Log IFN-γ rSP03B and log IFN-γ SGH (p=0.0004). Interestingly, a statistically significant correlation appears between age and log IFN-γ LSA.

Table 3. Relationship between log IFN-γ concentration and other quantitative clinical parameters using Spearman rho correlation and linear regression.

	Log IFN-γ concentration values					
Clinical Parameters	**IFN-γ LSA**		**IFN-γ SGH**		**IFN-γ rSP03B**	
	r_2	**p-value**	**r_2**	**p-value**	**r_2**	**p-value**
Log *Leishmania* specific antibodies	0.2708	0.0121*	0.1446	0.1866	0.0124	0.9098
Log IFN-γ SGH	0.0215	0.8445				
Log IFN-γ rSP03B	0.0732	0.5051				
Log IFN-γ rSP03B			0.3740	0.0004***		
Age (months)	0.2726	0.0121*	0.0481	0.6639	0.0862	0.4351

Log10 transformations were performed for each variable involving ELISA units (salivary antigens, *Leishmania* antigen, and IFN-γ antigen).

Significant p-values are designated with an asterisk (*p<0.05, **p<0.01, ***p<0.001).

Abbreviations: ELISA, enzyme-linked immunosorbent assay; IFN-γ, interferon gamma; LSA, *Leishmania* soluble antigen; SE, standard error, SGH, salivary gland homogenate.

Proportion of positive results of diagnostic tests studied and median results of all dogs studied

Table 4a summarizes the proportion of positive results for the six tests performed in Ibizan Hounds and other breeds as well as total dogs studied. In none of these tests was detected a significant difference in proportion of positives between Ibizan Hounds and other dog breeds (Fisher´s exact test: p-value >0.05).

Table 4a. Proportion of positive dogs for any of the diagnostic tests used.

Test	Total proportion of positives (n=85)	Proportion of positive Ibizan Hounds (n=51)	Proportion of positive of other breeds (n=35)	Fisher´s Exact test p-value
***Leishmania*-specific antibodies**	35.29% (30/85)	31.37% (16/51)	41.17% (14/34)	0.5558
SGH IgG	61.17% (52/85)	62.74% (32/51)	58.82% (20/34)	0.8211
rSPO3B IgG	45.88% (39/85)	49.00% (25/51)	41.20% (14/34)	0.5123
IFN-γ LSA	75.29% (64/85)	78.40% (40/51)	70.60% (24/34)	0.4494
IFN-γ SGH	40% (34/85)	35.29% (18/51)	47.05% (16/34)	0.3666
IFN-γ rSP03B	29.41% (25/85)	27.45% (14/51)	32.35% (11/34)	0.6360

Fisher´s exact test was conducted as two-tailed analysis.

Abbreviations: ELISA, enzyme-linked immunosorbent assay; IFN-γ, interferon gamma; LSA, *Leishmania* soluble antigen; SE, standard error, SGH, salivary gland homogenate.

Table 4b. Median and interquartile results of all dogs studied

Test	Ibizan Hounds	Other Breeds	Mann-Whitney p-value	All dogs
***Leishmania* ELISA**	25.96 EU (24.61 EU)	24.58 EU (58.10 EU)	0.8260	25.96 EU (35.48 EU)
SGH ELISA	48.10 EU (26.61 EU)	45.60 EU (58.10 EU)	0.8051	47.31 EU (30.13 EU)
rSPO3B ELISA	50.81 EU (50.24 EU)	36.56 EU (30.77 EU)	0.0313*	46.75 EU (45.26 EU)
IFN-γ LSA	1015.59 pg/ml (2122.92 pg/ml)	445.75 pg/ml (2336.95 pg/ml)	0.2695	854.40 pg/ml (2298.3 pg/ml)
IFN-γ SGH	24.77 pg/ml (31.36 pg/ml)	39.05 pg/ml (106.95pg/ml)	0.6212	26.75 pg/ml (68.56 pg/ml)
IFN-γ rSP03B	18.39 pg/ml (37.85 pg/ml)	28.60 pg/ml (39.35 pg/ml)	0.4342	19.25 pg/ml (44.99 pg/ml)

Abbreviations: ELISA, enzyme-linked immunosorbent assay; IFN-γ, interferon gamma; LSA, *Leishmania* soluble antigen; SE, standard error, SGH, salivary gland homogenate.

Interquartile values are expressed inside brackets.

Table 4b summarizes median and interquartile results obtained during this study of the different tests performed for Ibizan Hounds and dogs of other breeds. A significant statistical difference for median units only was obtained for rSP03B ELISA.

Interestingly, significant statistical differences were obtained when comparing IFN-γ LSA with IFN-γ SGH median concentrations (Mann-Whitney: p< 0.001) and also between IFN-γ LSA with IFN-γ rSPO3B median concentrations (Mann-Whitney: p< 0.001)(Values not reflected on table 4a).

Relationship between clinical parameters, P. perniciosus salivary antibodies and IFN-γ concentrations

Breed

The response of Ibizan Hounds and other breeds of dogs was evaluated and compared for both tests of *P. perniciosus* salivary antibodies and also IFN-γ values for LSA, SGH and rSP03B. Table 5 summarizes data related to SGH ELISA, showing that no statistical difference was found in proportion of dogs testing positive for Ibizan Hounds and other breeds. Median ELISA units showed no statistical difference neither for both groups. On the other hand, as pictured in Table 6, significant difference was found between both groups for rSP03B ELISA units, with median values higher for Ibizan Hounds (50.81 EU) than other breeds (36.56 EU) (Mann-Whitney: p= 0.0313). Although no statistical differences were found for proportion of positive counts (Fisher´s exact test: p= 0.5123).

IFN-γ LSA, as seen in Table 7, showed no statistical difference in median concentrations for both groups, although Ibizan Hounds presented higher values (1015.59 pg/ml) compared to other breeds (445.75 pg/ml) and no differences in proportion of positives between groups was observed.

In tables 8 and 9, median concentration values and Mann-Whitney p-values were calculated for all clinical parameters with the values obtained only from producer dogs, as non-producers for IFN-γ SGH accounted for the 60% (51/85) of the total of dogs and non-producers for IFN-γ rSP03B were 70.5% (25/60). Median values calculated from all dog would give values around 0, so that was avoided.

As seen on Table 8, only 35.3% of Ibizan hounds and 47.1% of other breeds were positive for IFN-γ SGH without statistically significant percentage of positives between

breeds. Median values were also lower for Ibizan Hounds (24.77 pg/ml) compared to other breeds (39.05 pg/ml). Similarly, as pictured in Table 9, median IFN-γ rSP03B

Table 5. Comparison of SGH ELISA units and proportion of positive results between dogs based on clinical parameters

		SGH ELISA units		SGH ELISA result	
Variable	**N(dogs)**	**Median ELISA units (IQR)**	**Mann-Whitney p-value**	**Proportion positive (count)**	**Fisher´s exact test p-value**
Breed					
Ibizan Hounds	51	48.10 (26.61)	0.8051	32/50, 64%	0,8211
Other Breeds	34	45.60 (58.10)		20/34, 58.8%	
Sex					
Female	60	47.06 (26.44)	0.6595	37/60, 61.7%	1
Male	24	50.66 (56.66)		15/24, 62.5%	
Age					
Young	34	47.41 (23.27)	0.7394	22/34, 64%	0,6503
Adult	50	46.71 (38.12)		29/50, 58%	
***Leishmania* ELISA**					
Positive	30	54.42 (38.27)	0.0029**	25/30, 83.3%	0.0023**
Negative	55	36.35 (31.93)		27/55, 49.1%	
IFN-γ LSA ELISA					
Producer	75	43.79 (28.48)	0.0707	44/75, 58.5%	0.3034
Non-Producer	10	55.6 (28.85)		8/10, 80%	
IFN-γ SGH ELISA					
Producer	34	39.47 (30.8)	0.163	18/34, 52.9%	0.2567
Non-Producer	51	49.88 (43.36)		34/51, 66.7%	
IFN-γ rSP03B ELISA					
Producer	25	36.6 (33.20)	0.1387	11/25, 44%	0.0506
Non-Producer	60	49.74 (30.49)		41/60, 68.3%	
rSP03B ELISA					
Producer	39	60.36 (53.37)	<0.0001***	35/39, 89.7%	<0.0001***
Non-Producer	46	33.28 (27.50)		17/46, 37%	

Mann-Whitney tests were conducted as two-tailed analysis.

Significant p-values are designated with an asterisk (*p<0.05, **p<0.01, ***p<0.001).

Abbreviations: ELISA, enzyme-linked immunosorbent assay; IFN-γ, interferon gamma; LSA*, Leishmania* soluble antigen; SE, standard error, SGH, salivary gland homogenate.

values were also lower for Ibizan Hounds (18.39 pg/ml) than other breeds (28.60 pg/ml) and proportion of positive results were equally lower for Ibizan Hounds than other breeds, although not statistically significant.*Sex*

As seen in Tables 5 and 6, no statistically significant differences were observed from median ELISA units between sexes for either *P. perniciosus* salivary antigens. No statistically significant differences were found for the proportion of positive counts in either test. Equally, no significant differences were detected between male and female dogs when evaluating IFN-γ concentration for LSA, SGH and rSP03B about median concentrations and proportion of positive counts (Tables 7, 8, 9).

Age

No statistically significant differences were observed for SGH and rSP03B median ELISA units for males and females (Tables 5 and 6) nor statistical differences either in the proportion of positive counts for these two parameters.

Interestingly, a statistical difference was observed for median values for IFN-γ LSA, as pictured in table 7, with young dogs showing a median value (388.40 pg/ml) much lower than adults (1381.18 pg/ml) (Mann-Whitney: p=0.0031). Proportion of positive results was also higher in adult dogs (82% vs.64.7%), although did not reach statistical significance.

When evaluating response for IFN-γ SGH and IFN-γ rSP03B, no statistical differences were found between young and adult dogs in mean concentrations or proportion of positives in either test. Interestingly, although statistical significance was not reached, mean values where higher for adult dogs for IFN-γ SGH, but adults had lower mean values for IFN-γ rSP03B (Tables 8 and 9).

Leishmania infantum serological status

As pictured in Tables 5, a statistical difference in proportion of positives for SGH was observed according to *L. infantum* serological status, with 83.3% of dogs positive to *Leishmania* serology also showing raised levels of SGH antibodies (Fisher´s exact test: p= 0.0023). A statistically significant difference in SGH median ELISA units was also observed between positive and negative dogs for *L. infantum* serology, with positive

dogs having higher SGH median values than negative dogs (54.42 vs. 36.35 EU) (Mann-Whitney: p=0.0029). Similar results were obtained for rSP03B, although without raising statistical significance on p-values (Table 6).

Table 6. Comparison of rSP03B ELISA units and proportion of positive results between dogs based on clinical parameters

		rSP03B ELISA units		rSP03B ELISA result	
Variable	**N(dogs)**	**Median ELISA units (IQR)**	**Mann-Whitney p-value**	**Proportion positive (count)**	**Fisher´s exact test p-value**
Breed					
Ibizan hounds	51	50.81 (50.24)	0.0313*	25/51, 49%	0.5123
Other breeds	34	36.56 (30.77)		14/34, 41.2%	
Sex					
Female	60	46.74 (45.55)	0.7702	26/60, 43.3%	0.4687
Male	24	56.65 (47.87)		13/24, 54.2%	
Age					
Young	34	53.24 (50.26)	0.1591	17/34, 50%	0.8152
Adult	50	43.51 (43.14)		22/50, 44%	
***Leishmania* ELISA**					
Positive	30	56.65 (48.42)	0.1086	17/30, 56.7%	0.1743
Negative	55	41.63 (43.47)		22/55, 40%	
IFN-γ LSA					
Producer	64	46.76 (42.27)	0.8505	29/64, 45.3%	1
Non-Producer	21	46.75 (57.80)		10/21, 47.6%	
IFN-γ SGH					
Producer	34	41.96 (45.04)	0.6505	15/34, 44.1%	0.8893
Non-Producer	51	46.78 (43.25)		24/51, 47.1%	
IFN-γ rSP03B					
Producer	25	39.07 (26.13)	0.3135	9/25, 36%	0.3396
Non producer	60	53.24 (43.97)		30/60, 50%	
SGH ELISA					
Producer	52	71.51 (53.71)	<0.001***	46/52, 88.5%	<0.001***
Non-Producer	33	31,96 (11.54)		15/33, 45.5%	

Mann-Whitney tests were conducted as two-tailed analysis.

Significant p-values are designated with an asterisk (*p<0.05, **p<0.01, ***p<0.001).

Abbreviations: ELISA, enzyme-linked immunosorbent assay; IFN-γ, interferon gamma; LSA, Leishmania soluble antigen; SE, standard error, SGH, salivary gland homogenate.

Table 7. Comparison of IFN-γ LSA ELISA units and proportion of positive results between dogs based on clinical parameters

		IFN-γ LSA concentration (pg/ml)		IFN-γ LSA result	
Variable	**N(dogs)**	**Median ELISA units (IQR)**	**Mann-Whitney p-value**	**Proportion positive (count)**	**Fisher´s exact test p-value**
Breed					
Ibizan hounds	51	1015.59 (2122.92)	0.2695	40/51, 78.4%	0.4494
Other breeds	34	445.75 (2336.95)		24/34, 70.6%	
Sex					
Female	60	801.00 (2093.96)	0.4911	46/60, 76.7%	1
Male	24	1101.15 (3512.73)		18/24, 75.0%	
Age					
Young	34	388.40 (1276.59)	0.0031**	22/34, 64.7%	0.1222
Adult	50	1381.18 (2949.48)		41/50, 82.0 %	
Leishmania ELISA					
Positive	30	1521.96 (2444.28)	0.0985	26/30, 86.7%	0.1133
Negative	55	731.85 (1797.05)		38/55, 69.1%	
IFN-γ SGH					
Producer	34	626.4 (2091.96)	0.6374	24/34, 70.6%	0.4494
Non-Producer	51	888.25 (2341.00)		40/51, 78.4%	
IFN-γ rSP03B					
Producer	25	1172.50 (2389.61)	0.3466	21/25, 84.0%	0.2798
Non producer	60	784.95 (2190.08)		43/60, 71.7%	
SGH ELISA					
Producer	52	1255.70 (1835.57)	0.0977	40/52, 76.9%	0.7971
non-Producer	33	556.3 (1193.79)		24/33, 72.7%	
rSP03B ELISA					
Producer	39	1181.00 (3274.19)	0.2069	35/39, 76.1%	1
Non-Producer	46	775.45 (1325.28)		29/46, 74.4%	

Mann-Whitney tests were conducted as two-tailed analysis.

Significant p-values are designated with an asterisk (*p<0.05, **p<0.01, ***p<0.001).

Abbreviations: ELISA, enzyme-linked immunosorbent assay; IFN-γ, interferon gamma; LSA, Leishmania soluble antigen; SE, standard error, SGH, salivary gland homogenate.

No statistically significant results were obtained for IFN-γ LSA, IFN-γ SGH and IFN-γ rSP03B when compared to positivity for *Leishmania* specific antibodies (Tables 7, 8, 9).

IFN-γ LSA

As depicted in Table 6, IFN-γ LSA producers had a trend to have lower median SGH ELISA units (43.79 EU) than non-producers (55.60 EU) (Mann-Whitney: p=0.0707). Proportion of positive counts did not reach statistical significance. No statistical significance was observed either between IFN-γ LSA and rSP03B (Table 7), IFN-γ LSA and IFN-γ SGH (Table 8) or IFN-γ LSA and IFN-γ rSP03B (Table 9).

IFN-γ SGH

No statistical differences were found between median SGH or rSP03B ELISA units for IFN-γ SGH producers and non-producers (Tables 5 and 6). No relationship was proved between production of IFN-γ SGH and IFN-γ LSA (Table 7). Statistical differences were observed for median values of IFN-γ rSP03B for IFN-γ SGH producers (35.3) and non-producers (2.62) (Mann-Whitney, p=0.0192) with a significant higher proportion of positives amongst the producers (44.1%) than the non-producers (19.6%) (Fisher´s exact test, p=0.0275) (Table 9).

IFN-γ rSP03B

Differences were observed in proportion of positives for SGH ELISA between IFN-γ rSP03B producers (44%) and non-producers (68.3%) (Fisher´s exact test, p=0.0506) although no statistically differences were observed in median ELISA units for SGH (Table 5). No significance was obtained for rSP03B (Table 6). No statistical difference was found related to IFN-γ LSA production between IFN-γ rSP03B producers and non-producers (Table 7). A significant difference in proportion of positive counts was observed for IFN-γ SGH amongst IFN-γ rSP03B producers (60%) and non-producers (31.7%) (Fisher´s exact test, p=0.0275), although median values did not reach statistical significance (Table 8).

Table 8. Comparison of IFN-γ SGH ELISA units and proportion of positive results between dogs based on clinical parameters.

		IFN-γ SGH concentration (pg/ml)		IFN-γ SGH result	
Variable	**N(dogs)**	**Median ELISA units (IQR) POSITIVES**	**Mann-Whitney p-value**	**Proportion positive (count)**	**Fisher´s exact test p-value**
Breed					
Ibizan hounds	51	24.77 (31.36)	0.6212	18/51, 35.3%	0.3666
Other breeds	34	39.05 (106.95)		16/34, 47.1%	
Sex					
Female	60	26.21 (32.47)	0.1913	22/60, 36.7%	0.3271
Male	24	77 (127.63)		12/12, 50%	
Age					
young	34	16.29 (48-19)	0.6063	12/34, 35.3%	0.5001
Adult	50	29.74 (62.00)		22/50, 44.0%	
Leishmania ELISA					
Positive	30	23.02 (27,73)	0.1532	11/30, 36.7%	0.8171
negative	55	32.50 (96.60)		23/55, 41.8%	
IFN-γ LSA					
Producer	64	27.02 (82.10)	0.8381	24/64, 37.5%	0.4494
Non-Producer	21	26.44 (30.95)		10/21, 47.6%	
IFN-γ rSP03B					
Producer	25	32.5 (22.6)	0.3538	15/25, 60%	0.0275*
Non producer	60	21.33 (91.92)		19/60, 31.7%	
SGH ELISA					
Producer	52	26.75 (70.71)	0.6212	18/52, 34.6%	0.2576
Non-Producer	33	26.70 (50.79)		16/33, 48.5%	
rSP03B ELISA					
Producer	39	36.65 (79.14)	0.1196	15/39, 38.5%	0.8272
Non-Producer	46	23.02 (36.34)		19/46, 41.3%	

Mann-Whitney tests were conducted as two-tailed analysis.

Significant p-values are designated with an asterisk (*p<0.05, **p<0.01, ***p<0.001).

Abbreviations: ELISA, enzyme-linked immunosorbent assay; IFN-γ, interferon gamma; LSA, *Leishmania* soluble antigen; SE, standard error, SGH, salivary gland homogenate.

Table 9. Comparison of IFN-γ rSP03B concentration and proportion of positive results based on clinical parameters.

		IFN-γ rSP03B concentration (pg/ml)		IFN-γ rSP03B result	
Variable	**N(dogs)**	**Median values (IQR) POSITIVES**	**Mann-Whitney p-value**	**Proportion positive (count)**	**Fisher´s exact test p-value**
Breed					
Ibizan hounds	51	18.39 (37.85)	0.4342	14/51, 27,5%	0.636
Other breeds	34	28.60 (39.35)		11/34, 32.4%	
Sex					
Female	60	33.11 (45.57)	0.4867	16/60, 26.7%	0.4287
Male	24	18.5 (26.2)		9/24, 37.5%	
Age					
Young	34	27.20 (40.27)	0.9773	8/34, 23.5%	0.3404
Adult	50	19.25 (36.27)		17/50, 34%	
***Leishmania* ELISA**					
Positive	30	18.5 (37.80)	0.8931	11/30, 36.7%	0.3238
negative	55	27.27 (37.80)		14/55, 25.5%	
IFN-γ LSA					
Producer	64	19.25 (45.80)	0.8027	21/64,32.8%	0.2798
Non-Producer	21	29.47 (25.14)		4/21, 19%	
IFN-γ SGH					
Producer	34	35.3 (58.88)	0.0192*	15/34, 44.1%	0.0275*
Non-producer	51	2.62 (26.07)		10/51, 19.6%	

Mann-Whitney tests were conducted as two-tailed analysis.

Significant p-values are designated with an asterisk (*p<0.05, **p<0.01, ***p<0.001).

Abbreviations: ELISA, enzyme-linked immunosorbent assay; IFN-γ, interferon gamma; LSA, *Leishmania* soluble antigen; SE, standard error, SGH, salivary gland homogenate.

Discussion

Canine leishmaniosis is a neglected zoonosis transmitted by sand fly bites, where susceptibility and affectation are linked to complex Interactions between host and parasites (Rohoušová & Volf, 2006). Sand fly saliva can modulate the host immune response at innate and acquired level, with a wide range of effects observed, where host´s immune response will be determinant over clinical manifestations and progress

of the disease (Hosein et al., 2017; Lestinova et al., 2017). This study examines the relation between humoral and cellular immune responses. To the best author´s knowledge, this is the first study investigating canine cellular immune response against *P. perniciosus* salivary proteins in dogs living in an endemic area for leishmaniosis. To measure cellular and humoral immunity, an IFN-γ release whole blood assay was stimulated with three conditions: *Leishmania* soluble antigen (LSA), salivary gland homogenate (SGH) and recombinant salivary protein rSP03B. In addition, antibodies against SGH and rSP03B were also measured. rSP03B is a universal marker for *P. perniciosus* exposure (Kostalova et al., 2017). Moreover, Its good correlation with antibodies againstSGH has been seen in experimentally exposed hosts and natural infections (Lestinova et al., 2017).

Several studies were performed in the past to explore immunity paths against canine leishmaniosis, where cellular response was correlated to clinicopathological, immunological and parasitological findings (Collin et al., 2009; Montserrat-Sangrà et al., 2018; Ordeix et al., 2019; Burnham et al., 2020). The present study combines measurements from two groups of dogs from Mallorca, one composed of Ibizan hound and the other group with dogs of other breeds. Previous studies pointed to an increased cellular immunity in Ibizan hounds (Solano-Gallego et al., 2000). In this study, interesting agreements were found between salivary antibody levels, IFN-γ concentrations related to salivary and *Leishmania* antigens and *Leishmania*-specific antibody levels.

In humans, a similar study was performed to explore cellular and humoral responses to *P. papatasi* in an endemic areas of different prevalence for *L. major* in Tunis, and concluded that individuals frequently exposed to sand fly bites develop higher anti saliva IgG responses and IFN-γ levels, even so, less than 30% of the individuals expressed proliferation against salivary gland extracts on blood cultures, regardless of immune status or living area (Kammoun-Rebai et al., 2017). When examining relations between IFN-γ LSA, IFN-γ SGH and IFN-γ rSP03B, a slight agreement between IFN-γ LSA and IFN-γ rSP03B (44.71%) was found in the present study. However, a significant and clear association between antibodies against salivary antigens, IFN-γ production after stimulation with salivary antigens and *L. infantum* infection was not observed in our

study, even if the tests used are quite comparable between them, as previously described in humans (Kammoun-Rebai et al., 2017). Nevertheless, according to our present study, around 40% of the total of dogs were showing production for IFN-γ SGH and 29.4% for IFN-γ rSP03B. Furthermore, a fair agreement was found between IFN-γ SGH and IFN-γ rSP03B (65.88%). Spearman correlation was also significant for log IFN-γ rSP03B units and log IFN-γ SGH units ($p<0.001$). Significant statistical differences in proportion of positive counts was observed for IFN-γ SGH production amongst IFN-γ rSP03B producers and non-producers and vice versa. Around 17.6% (15/85) of the total of dogs of this study produced both types of IFN-γ. In addition, a low concentration of IFN-γ was found in the present study when stimulated with SGH or rSP03B when compared with LSA, as previously reported in humans (Kammoun-Rebai et al., 2017). A hypothesis to explain these low amounts of IFN-γ SGH and IFN-γ rSP03B would be as a mechanism to protect the body against hyper reactions in moments of high exposure to sand fly bites. Instead, the immune system would mount a higher humoral reaction towards sand fly saliva. Nevertheless, cellular immunity against the parasite seems to be a good strategy, as higher amounts of IFN-γ LSA appear to be a protective factor against disease.

When it comes to salivary antibody levels, significant percentage of agreement (75.79%) was found between SGH ELISA and rSP03B ELISA salivary tests, with a kappa value of 0.515±0.088 and strong positive correlation on their log units. These values are very similar to those found in a previous study also set in Mallorca (Burnham et al., 2020), where diagnostic performance of these same tests reached a 75.76%. Similar agreement between SGH ELISA and rSP03B ELISA salivary tests was also found in other previous studies located in Italy and Portugal (Kostalova et al., 2015; Kostalova et al., 2017). In our study, we observed a significant difference in the proportion of positive responses from SGH ELISA and rSP03B ELISA ($p=0.0088$), with a 61.17% of positives for SGH ELISA compared to 45.88% of positives for rSP03B ELISA. This difference could be attributed to the use of different antigens to perform the test: SGH includes a wide range of salivary proteins found in native forms, with a composition that could vary depending on colony factors. Therefore, SGH could have antigenic properties with a potential to cause cross reactions with other molecules (Lestinova et al., 2017) while

recombinant proteins, such as rSP03B used in the present study, are more specific but less sensitive.

One of the hypotheses of this study was that Ibizan hounds would have higher levels of anti-*P. perniciosus* saliva IgG antibodies than dogs of other breeds due to increased exposure to sand fly bites. After comparing median ELISA units for SGH, Ibizan hound had a median of 48.10 EU and dogs of other breeds 45.60 EU, so we did not find statistically significant differences for both groups of dogs. Conversely, statistically significant differences were found for rSPO3B ELISA units for both groups, with a median of 50.81 EU for Ibizan Hounds and 36.56 EU for dogs of other breeds. In a previous study also set in Mallorca and very similar groups of dogs (Burnham et al., 2020), statistical differences were found for Ibizan Hounds in median SGH units but not for rSP03B. A detail to compare from both studies are the median values obtained in these parameters: in a previous study, blood samples were taken in December and median values observed for SGH and rSP03B ranged between 25-30 EU, whilst in the present study, were blood samples were taken in September, the same values ranged between 36-50 EU approximately. That could agree with the fact that antibody levels against sand fly saliva fluctuate along the year according the abundance of vectors and sand fly bites (Kostalova et al., 2015; Alten et al., 2016). In Mallorca island, due to its location on the Mediterranean Sea, there is an estimated beginning of sand fly season ranging from April to first half of June, with a wide peak of density usually falling between July and September. Adult sand flies end activity from mid-September through November, with apparent no risk of leishmaniosis transmission between December and March (Alten et al., 2016). According to a study in North-West Spain, associations were found between vector exposure and salivary antibody levels, finding the lowest levels during winter months (Velez et al., 2018).

Fair agreement (62.35%) was found between SGH ELISA salivary tests and *Leishmania*-specific antibody test, and fair agreement between *Leishmania*-specific antibody test and rSP03B ELISA (47.06%). Significant Spearman´s correlation was also found between log ELISA units for *Leishmania*-specific antibodies for both SGH (p=0.0001) and rSP03B (p=0.0011). Correlation was observed as well between log SGH ELISA values and IFN-γ rSP03B concentration units (p= 0.0361). Moreover, positive dogs for *Leishmania*-

specific antibody test showed higher proportion of positives for SGH ELISA (83.3% vs. 49.1%) and had higher median ELISA units than negative dogs (54.42 EU vs. 36.35 EU), Similarly, positive dogs for *Leishmania*-specific antibody test had a trend to be also positive for rSP03B ELISA (56.7% vs. 40%) and have higher median units than negatives (56.65 vs 41.63 EU). This results are similar to previously reported in other studies where seropositive dogs for *Leishmania* show also raised amounts of anti-saliva antibodies (Quinnell et al., 2018; (Maia et al., 2020)

Interestingly, IFN-γ LSA is also showing a 50.5% of agreement with *Leishmania*-specific antibody test and 57.65% for SGH ELISA. Spearman correlation was found for log IFN-γ LSA units and *Leishmania*-specific antibody test. Furthermore, there is a significant relationship between IFN-γ LSA and age when comparing log concentration units of between IFN-γ LSA and months of age, as median values increased in older dogs. Relation of IFN-γ LSA and clinical disease has been already explored in previous studies, where a low production of IFN-γ LSA is associated to clinical disease and unfavorable outcomes (Martínez-Orellana et al., 2017; Solano-Gallego et al., 2016).

Even so, in none of the test performed was detected a significant difference in proportion of positives between breed groups. A possible explanation of lack of significant differences between groups could be because samples were taken in September, a time of the year were a raised immune response is expected from all dogs after several months exposure to sand flies. In a previous study with similar characteristics where Ibizan hounds and dogs of other breeds were compared (Burnham et al., 2020), a significant proportion of dogs testing positive for IFN-γ LSA was found. As previously said, that assay was performed taking the blood samples in December, when low levels of immunity are expected and maybe differences between groups would become more apparent, maybe that would explain the results obtained. Levels of immunity fluctuate along the year so is expected to find high values of immunity markers during warmer seasons and lower levels in winter (Kostalova et al., 2015; Alten et al., 2016).

Again comparing results with a previous study (Burnham et al., 2020), in both cases the groups composed with Ibizan hounds had younger median ages than the groups

composed by other breeds, so lower levels of immunity would be expected from young dogs and that could have had an influence over the results of both studies, as age is a factor expected to be related to sandfly exposure. Overall exposure to sand flies increases the older is the dog. Interestingly, in this study, a statistical difference was observed related to age for median values of IFN-γ LSA, with young dogs showing values (388.40 UE) much lower than adults (1381.18 EU). Proportion of positive results was also higher in adult dogs (82% vs.64.7%), although this fact did not reach statistical significance. In this study, age did not seem to have a clear influence over mean concentration values or proportion of positives on IFN-γ SGH and IFN-γ rSP03B concentrations, but as previously stated, the percentage of positives to these tests was relatively low, making the sample too small to take conclusions. The size of the sample may have played a role on the overall of the study and could be considered not large enough to be fully representative.

Many of the results obtained here reflected other previous studies from other authors, were a clear relation is established between *P. perniciosus* saliva and immune responses against *Leishmania* infection (Andrade & Teixeira, 2012). In this study, a connection have been found between IFN-γ SGH, IFN-γ rSP03B and all the other parameters evaluated, opening a door for further investigations to know better their role inside the cellular mechanisms of immunity, including comparisons with other markers of immunity and disease. Furthermore, this study was performed with dogs enrolled from an endemic area of canine leishmaniosis, living in the same area and the same environmental conditions, mainly seronegative or low positive for *Leishmania*. Should dogs be strong positive of come from a non-endemic area, results maybe would have been different, also from samples taken in different moments of the year, so plenty of possibilities are open to continue researching.

There is a growing interest for salivary markers of vectors, as salivary proteins have been investigated as a novel component for the new generation of vaccines against *Leishmania* (Moreno, 2019). Also, they are used to assess risk of disease transmission and as a tool to evaluate vector control campaigns. Use of short peptides based on salivary antigens could be replacing whole salivary gland homogenate (SGH) or recombinant proteins, to avoid some of their disadvantages (Sima et al., 2019).

In conclusion, dogs living in endemic areas for leishmaniosis present cellular and humoral immunity against *P. perniciosus* salivary proteins. Low concentrations of IFN-γ SGH and IFN-γ rSP03B are found in less than 20 % of dogs, in contrast with higher levels of IFN-γ LSA and IgG anti salivary gland homogenate and recombinant protein rSP03B. Higher levels of IFN-γ LSA also seem to be associated to age and *Leishmania*-specific antibodies.

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