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Title: Comparative analysis of the *Leishmania infantum*-specific antibody repertoires and the autoantibody repertoires between asymptomatic and diseased dogs

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Highlights

- Dogs with cVL demonstrate higher titers of specific Tot IgG, IgG1 and IgG2.
- The production of autoantibodies starts since the subclinical phase of dogs' infection.
- Autoreactive IgG1 dominates the autoantibody repertoire of asymptomatic dogs.
- Cross-reactive IgG1 antibodies dominate the autoantibody repertoire of sick dogs
- Anti-transferrin autoantibodies are produced during both stages of dog's infection.

ABSTRACT

Leishmania (*L.*) *infantum*-infected dogs may present with a large range of clinical signs, from apparently healthy with no or few (asymptomatic dogs, AD) to several clinical signs characteristic of active infection (symptomatic dogs, SD). The present study is justified by the conflicting reports describing that either *L. infantum*-specific IgG1 or IgG2 antibodies may be used as isotype marker of the asymptomatic infection status and by the lack, to our knowledge, of previous analysis of the IgG sub-classes autoantibody repertoires of *Leishmania*-infected dogs. On the basis of clinical evaluation and laboratory testing (IFAT, parasitological examination of Giemsa-stained lymph node smears, *L. infantum* antigens-ELISA of total (Tot) IgG), 131 dogs were categorized as AD, SD, healthy non infected dogs (HND) from surrounding areas, and as negative control dogs (CTD). ELISA based on leishmanial native antigens or recombinant LACK and LeIF proteins showed that SD produce higher levels of specific Tot IgG, IgG1 and IgG2 antibodies than AD, and that for both clinical stages, the antibody titers of IgG2 isotype were constantly higher than those of the IgG1. The seroprevalences of Tot IgG, IgG2 did not differ between AD and SD groups (97 and 97% in SD; 100 and 96% in AD, respectively) whereas that of IgG1 was slightly lower in SD (88% of AD versus 82% of SD). The autoantibody repertoires were analyzed by ELISA using HEp-2 extracts, ds-DNA, human albumin and transferrin as self-antigens and by Western blot using HEp-2 proteins.

ELISA results' indicated that AD develop higher levels of IgG1 autoantibodies, and higher seroprevalence (50% and 26% in AD and SD respectively), contrasting with lower levels and seroprevalences of Tot IgG and IgG2 (43 and 68% for AD; 100 and 74% for SD). Interestingly, SD showed a stronger IgG1 and particularly IgG2 reactivity with transferrin, an iron-binding protein, than AD and HND. Western blotting experiments produced heterogeneous IgG1 and IgG2 inter- and intra-groups reactivity profiles towards HEp-2 proteins, to identify a specific antigenic profile. Generated data from competitive HEp-2-ELISA using leishmanial antigens as inhibitors were in favor that IgG1 antibodies are predominantly autoantibodies to self-antigens in AD whereas they are mainly cross-reactive (*Leishmania*/self-antigens) autoantibodies in SD.

Key words: *Leishmania infantum*; Canine visceral leishmaniasis; IgG1 and IgG2 isotypes; Autoantibodies; Human transferrin.

1. Introduction

Domestic dogs are the primary reservoir hosts for zoonotic visceral leishmaniasis (VL), the most widespread entity of leishmaniasis caused by the protozoa *Leishmania* (*L.*) *infantum*. Due to the close proximity between dogs and humans, canine cases often precede the occurrence of human cases. The disease is endemic in the Mediterranean basin, Middle East, Central Asia and Latin America (Alvar et al., 2012). Previous investigations have categorized *L. infantum*-infected dogs into three clinical forms: (i) asymptomatic dogs (AD) with no suggestive signs of VL or other diseases; (ii) oligosymptomatic with a maximum of three clinical signs and (iii) symptomatic dogs (SD) with characteristic clinical signs of VL (Quinnel et al., 2003; Koutinas and Koutinas, 2014). The major signs of canine VL (cVL) include hepatosplenomegaly, lymphadenopathy, cutaneous lesions, keratoconjunctivitis, opaque bristles, alopecia, apathy, onychogriphosis, anorexia, anaemia and severe weight loss (Bettini and Gradoni, 1986; Abbehusen et al., 2017). The serological diagnosis of *L. infantum* infection in AD, potential carriers of *Leishmania* parasite, remains so far problematic and their living near human hosts constitute a serious issue for public health.

Laboratory findings showed that cVL is characterized by the suppression of the cellular immune responses to specific antigens and as the disease progresses to mitogens, as well as a strong up-regulation of specific and "non specific" humoral responses (Pinelli et al., 1994; Cabral et al., 1998). The main hypothesis that were generally retained to explain hypergammaglobulinemia induction and autoantibodies appearance are that (i) parasite-derived antigens may activate T helper (Th) 2 lymphocytes for the production of B-cell stimulating interleukins (IL) such as IL-4, IL-6 and IL-10; (ii) parasite antigens may directly act as mitogenic substances to B cells; (iii) *Leishmania* parasite induces by cell damage and tissue destruction the release of host's autoantigens which in turn causes autoreactivity; (iv) molecular mimicry between *Leishmania* antigens and host's self-antigens yields also to B cells activation. Polyclonal activation of B cells results in an increase of the levels of non specific antibodies including natural antibodies, the concentration of circulating immune complexes and to a lesser extent, the levels of *Leishmania*-specific antibodies (Greenwood,

1974; Pateraki et al., 1983; Galvão-Castro et al., 1984; Böhme et al., 1986; Argov et al., 1989; Louzir et al., 1994; Liberopoulos et al., 2003; Montes et al., 2007; Tunccan et al, 2012; Jamal et al., 2017).

Numerous investigations of human, mouse and dog models have attempted to determine the significance and prognostic value of the *Leishmania*-specific IgG subclasses in parasite-infected hosts (Anam et al., 1999; Bhattacharyya et al., 2014; Sassi et al., 2015; Day et al., 2007). Indeed, the levels of specific IgG1 and IgG2 antibodies directed towards crude, soluble promastigote or amastigote lysates, were extensively investigated by enzyme-linked immunosorbent assay (ELISA) and/or by western blotting (WB), in cases of symptomatic (SD) and asymptomatic dogs (AD) that have been naturally or experimentally infected with *L. infantum*. Conflicting results were reported, and it has been suggested that the lack of correlation between IgG subclasses and parasite infection stage could be due to a weak sensitivity of the used leishmanial antigens and/or of the anti-IgG subclasses commercial antibodies, i.e. polyclonal *versus* monoclonal antibodies (Quinnell et al., 2003; Day et al., 2007; Carson et al., 2010). Some studies associated asymptomatic infection with higher IgG2 levels compared to IgG1 (Deplazes et al., 1995; Iniesta et al., 2005), while others observed the opposite during active clinical infections (Bourdoiseau et al., 1997; Nieto et al., 1999; Boceta et al., 2000; Almeida et al., 2005; Reis et al., 2006; Cardoso et al., 2007; Teixeira Neto et al., 2010). Carson et al. (2010) described that the levels of both IgG subclasses are higher in SD whereas in a more recent study Lima et al. (2017) reported higher IgG1 levels for SD but no differences in those of IgG2 between AD and SD.

It is in this context we aimed the present study to (i) analyze the profile of specific IgG1 and IgG2 antibodies in a cohort of domestic dogs living in Tunisian endemic areas for *L. infantum* transmission, in order to check whether IgG1 or IgG2 may characterize dog's clinical status and (ii) investigate whether the production of autoantibodies played a role in the conflicting data reported in the literature.

2. Materials and methods

2.1. Animals and sera

The Ethical Committee of the Institute Pasteur of Tunis provided its approval for the study protocol. A total number of 131 dogs' sera were collected after the owners' consent. Sera were stored at −20°C until analyzed. Based on clinical examination, parasitological and serological analyses, three dog groups were constituted.

The first group consisted of 34 naturally infected sick dogs, none have received specific anti-*Leishmania* drugs. Dogs, originated from Bizerte, Tunis, Ariana, Manouba and Ben Arous districts, were presented to the Parasitology Department at the National School of Veterinary Medicine of Sidi Thabet (Tunis. Tunisia) between September 2009 and December 2011 for diagnostic purposes. Dogs were submitted to clinical examination and symptoms such as apathy, weight lost, skin lesions, lymph node and spleen enlargement, onychogryphosis and anemia, were recorded. For each dog, a score varying between zero (no sign) to three (severe) was attributed depending on the symptom intensity and a total clinical score (TCS) was attributed (Quinnell et al., 2003). The TCS ranged from 3 to 16 (mean 11.2 ± 3.6). A direct detection of *Leishmania* parasite, performed by the examination of Giemsa-stained lymph node aspirates, showed nine out of thirty-four (26%) negative dogs. All serum samples tested positive by the immunofluorescence antibody test (IFAT) (serum dilution > 1/80).

The second group consisted of 71 serum samples collected from healthy dogs during epidemiological investigations of Tunisian *L. infantum* endemic foci. Physical examination did not show any clinical symptoms that could be attributed to VL or any other diseases. This group was subdivided into two groups based on the reactivity of their Tot IgG towards *L. infantum* antigens: a group of healthy non-infected dogs (HND) with IgG reactivity lower than the cut-off value and a second group of asymptomatically infected dogs (AD) with IgG reactivity higher than the cut-off value.

The third group consisted of 26 archived negative serum samples obtained from healthy control dogs (CTD) with laboratory negative results. This group was used for the determination of ELISA cut-off values.

2.2 Antigens for immunoenzymatic assays

L. infantum (MHOM/TN/80/IPT-1) promastigotes were maintained in RPMI-1640 medium supplemented with 2 mM L-glutamine, 100 units/ml Penicillin, 100 µg/ml Streptomycin and 5% of heat-inactivated fetal bovine serum (FBS) at 26°C. Parasites were collected at the stationary phase, washed three times in cold phosphate buffered saline (PBS) pH 7.2 by centrifugation at 3,000 g for 15 min and then lysed in 10 mM Tris 1 mM EDTA (pH 8.0) PMSF 1 mM by six cycles of freezing at −80°C and thawing at room temperature (RT). Preparations were centrifuged at 12,500 rpm for 45 minutes at +4°C and the supernatant was harvested and stored at −70°C until use in ELISA experiments. Recombinant proteins, *Leishmania* homologue of receptors for activated C kinase (LACK) and *Leishmania* elongation initiation factor (LeIF), were kindly provided by C. Bahloul (Institut Pasteur de Tunis. Tunisia). Human albumin and human transferrin were purchased from Sigma (France).

Human epithelial (HEp-2) adherent cells were cultured in RPMI-1640 medium supplemented with 5% FBS, at 37°C and 5% CO_2 in a humidified incubator. Cells were grown to 5 x 10^5 cells/ ml then harvested by centrifugation for 5 min at 2000 rpm. Cells' pellets were washed three times with PBS and lysed on ice for 30 min in Tris-HCl 50 mM pH 8 containing 0.1% Triton X100. Supernatant was collected after centrifugation at 12,000 rpm for 30 min and used in ELISA for the detection of autoantibodies.

Protein concentrations of leishmanial antigens and HEp-2 preparations were determined by the BCA kit (Sigma) based on a bovine serum albumin standard curve.

2.3. Assessment of the Leishmania-specific antibody responses by ELISA

Ninety-six well microplates (MaxiSorp. Nunc) were coated with 100µl/well of leishmanial soluble antigens (5 µg/ml), LACK or LeIF recombinant proteins (2 µg/ml) diluted in bicarbonate buffer (0.1M NaH CO_3, pH 8.1). After overnight incubation at 4 °C, wells were washed twice with PBS containing 0.05% Tween-20 (PBS-Tw) and received 200µl of 2% skimmed milk (PBS-Tw-milk) for 1 H at 37°C to reduce non-specific binding. Blocking buffer was removed by two washes and dog's sera, diluted in PBS- Tw- milk as described in figure legends, were added to wells. Plates were incubated 1H at 37 °C then washed seven times. One hundred microliters of polyclonal peroxidase-conjugated anti-dog antibodies, diluted in PBS-Tw-milk, was added and incubated for 1H at RT. The plates were washed six times and the substrate ortho-phenylenediamine (0.4 mg/ml) dissolved in 100 mM citrate buffer pH 5.0 containing 0.03% hydrogen peroxide was added. Reactions were stopped by the addition of 50 µl/well of 3 N sulfuric acid and optical densities (OD) were recorded at 492 nm using an ELISA reader. The conjugates anti-dog IgG antibodies (Sigma), anti-IgG1, and anti-IgG2 (both from Bethyl Laboratories) were used at a dilution of 1:10,000, 1:2,000 and 1:20,000, respectively.

2.4. Assessment of the serum autoantibody levels by ELISA

The detection of IgG, IgG1 and IgG2 autoantibodies in dog's sera was performed by ELISA as described above using microtitre plates coated with HEp-2 extracts (Binder et al., 2005), with human albumin or human transferrin (5 µg/ml) or with double-stranded deoxyribonucleic acid (ds-DNA, 10 µg/ml), diluted in bicarbonate buffer. Dogs' sera were tested at dilutions given in figures' legends.

2.5. Analysis of the specificity of IgG1 and IgG2 autoantibodies by competitive ELISA

The specificity of the IgG1 or IgG2 binding to leishmanial antigens was examined by competitive ELISA. Dogs' serum samples obtained from 8 AD and 7 SD positive for reactivity with HEp-2 antigens were diluted at 1:150, 1:300, 1:600 and 1:1,200 then pre-incubated (v/v) for 3 to 4

H at 37°C with (W) 100 µg/ml of leishmanial antigens or without (WO, buffer only). The reactivity of IgG1 and IgG2 antibodies was measured, as described above, by ELISA on HEp-2 coated plates. Results were expressed as the mean OD ± 1 SEM of the antibody binding with or without inhibitor. Percentage of inhibition was determined as follows: 100-[OD_{W}/OD_{WO} x 100].

2.6. Western blotting

Proteins from HEp-2 lysates were separated by 15% SDS-PAGE under reducing conditions and transferred to nitrocellulose membranes (GE Healthcare, Buckinghamshire, UK). Nitrocellulose strips were placed in PBS, 0·1% Tween-20 (PBS-Tw), 2% skim milk and incubated overnight at 4°C. Incubation of nitrocellulose strips with dog sera, diluted in PBS-Tw-2% milk, was carried out during 1 H at RT with mechanical agitation. Goat anti-dog IgG1 (1/3,000) and sheep anti-dog IgG2 (1/25,000) both conjugated to HRP were incubated for 45 and 30 min at RT, respectively. Unbound antibodies were removed by extensive washing with PBS-Tw. Peroxidase enzymatic reactions were detected by an enhanced chemiluminescence (ECL) assay used according to the manufacturer's instructions (GE Healthcare, Buckinghamshire, UK). The molecular weight (MW) of the more reactive bands was determined in kDa using standard proteins of known MWs.

2.7. Statistical analysis

Data analyses were performed using the GraphPad Prism software. Cut-off values for positivity were defined as being two or three standard deviations (SD) from the mean absorbance of the negative control serum samples (CTD). Normality of the data was established using the Kolmogorov-Smirnoff test. Differences in the OD median or mean values of antibody reactivity between groups were evaluated in the 95% confidence interval using paired or unpaired Student's *t*-test and the Mann-Whitney-U test, as indicated in figure's legends. Differences between groups were considered significant when P values were < 0.05. Statistical associations were established using the Pearson's correlation coefficient (r).

3. Results

3.1. Anti-Leishmania Tot IgG, IgG1 and IgG2 levels in dogs' sera

The levels of Tot IgG and of two IgG subclasses to *L. infantum* extracts were measured for all study groups. Results, shown in Fig. 1A, indicate a stronger reactivity of Tot IgG and IgG2 towards leishmanial antigens in SD sera, comparatively to IgG1 antibodies ($P < 0{,}000001$). One dog (TCS = 13), positive by IFAT but negative by Giemsa staining test, did not show any significant serum antibody reactivity towards leishmanial antigens. Another dog (TCS = 7), positive by both tests, showed borderline Tot IgG and IgG2 ODs.

Within the HD group, sera from 36% (26/71) of dogs were positive for Tot IgG which suggests an asymptomatic infection. All demonstrated IgG2 antibody reactivity towards leishmanial antigens and only three presented an IgG1 reactivity lower than the cut-off value (Fig. 1A). Similarly to the SD group, Tot IgG and IgG2 antibodies were more reactive than IgG1 antibodies in AD. Interestingly, among the remaining 45 HND (negative for Tot IgG), 62% (27/45) of dogs were negative for both IgG isotypes while 31% (14/45) and 6.6% (3/45) of dogs showed, even though ODs were borderline, an exclusive IgG1 or IgG2 reactivity, respectively (Fig. 1A).

Comparison between AD and SD groups indicate, at both tested serum dilutions, lower levels of Tot IgG, IgG2 but also of IgG1 antibodies in AD (Fig.1A). Interestingly, the AD group displayed a slightly higher frequency of positive IgG1 reactivity comparatively to SD but only at 1:100 serum dilution (88% for AD *versus* 82% for SD). The production of Tot IgG in dog's sera correlated positively with that of IgG2 in both AD (at dilution 1:500, $r = 0.83$; $P < 0.0001$; Fig. 1B) and SD groups (at dilution 1:2,500, $r = 0.92$, $P < 0.0001$; Fig. 1B) whereas no significant correlation was observed with the IgG1 isotype ODs.

Considering the controversial findings regarding the IgG isotype profile associated with cVL and the results obtained in the present study showing that neither the reactivity of the IgG1 isotype nor that of the IgG2 with leishmanial antigens is relatively higher in AD than in SD, we opted to re-

analyze dogs' serum reactivity in order to determine their antibody titers. Serum samples from 12 AD and 16 SD, which presented high IgG1 and/or IgG2 reactivity were tested by ELISA at dilutions 100, 400, 1,600 and 6,400. Obtained data clearly confirms that SD produce higher serum levels of IgG1 and IgG2 comparatively to AD (Fig. 1C).

Most of the investigations of the humoral response of *L. infantum* naturally-infected dogs have used either whole promastigote extracts or soluble leishmanial antigens obtained after promastigote lysis/sonication followed or not by removal of insoluble material. The use of such antigenic preparations which contain nuclear and cytoplasmic components, conserved between species, could have resulted in non specific binding of IgG1 and/or IgG2 dog's antibodies. In order to check whether the use of *Leishmania*-specific antigens would generate results that corroborate those obtained herein with total soluble antigens, we assessed the reactivity of IgG isotypes using recombinant LACK (Fig. 2A and B) and LeIF (Fig. 2C and D) proteins. Dogs from HND were tested in parallel. Results indicate that IgG1 (Fig. 2.A and C) and IgG2 (Fig. 2.B and D) productions by AD and SD infected dogs were significantly different when compared with HND ($P < 0.02$). For both recombinant proteins, the IgG1 subclass levels and especially those of IgG2, were significantly higher in SD compared to AD sera (at dilution 1:400, $P = 0.0006$, data not shown), corroborating the data from native antigens-based ELISA. The frequency of positive reactions (Freq %) was constantly higher for SD group, for both isotypes and both recombinant proteins (Fig. 2).

3.2. Evaluation of Tot IgG, IgG1 and IgG2 autoantibodies reactivity of dog's sera by ELISA

Considering that (i) the conflicting results reported in the literature were often related to the IgG1 isotype where authors have considered it as a surrogate marker of the asymptomatic stage (ii) the relatively higher frequency of IgG1 binding to leishmanial antigens observed in AD (88 and 82% for AD and SD, respectively) despite a significant lower levels of specific IgG1, we hypothesized that their binding to leishmanial antigens could be non specific. Serum samples form 22 AD, 34 SD, and 27 HND were tested by HEp-2-ELISA at dilutions 1:100 and 1:200. Results

indicate the presence of Tot IgG, IgG1 and IgG2 autoantibody reactivity in the sera of AD and SD groups and at a lesser extent in HND (Fig. 3). The highest reactivity of Tot IgG and IgG2 was obtained for the SD group and all comparisons were statistically significant (Fig. 3). Moreover, we observed a significant difference at 1:100 dilution between the level of IgG1 autoantibodies in AD comparatively to HND (P = 0.017) but not to SD group. Interestingly, AD group demonstrated again an increased frequency of IgG1 antibodies binding to HEp-2 antigens (50% *versus* 26% considering the whole group and 60% *versus* 28% considering only IgG1-positive dogs in *L. infantum*-ELISA).

Tot IgG reactivity with HEp-2 antigens correlated positively with that of IgG2, in both AD and SD groups (Fig. 3B) whereas, as seen for leishmanial antigens, no significant association was obtained using IgG1 ODs at any tested serum dilution. Moreover, *L. infantum*-IgG2 ODs correlated with HEp-2-IgG2 ODs in SD (r = 0.41; P = 0.014) but not in AD (r = 0.35; P = 0.1; Fig. 3C).

Considering the heterogeneity of dog's antibody reactivity, sera from 5 SD and 6 AD, which showed higher than 3 SD of the mean of CTD group, were selected for more detailed analyses of their IgG1 and IgG2 subclass reactivity to HEp-2 antigens. Samples diluted at 1:100, 1:200, 1: 400 and 1:800 were tested by HEp-2 ELISA. Binding curves presented in Fig. 4 indicate a higher IgG1 reactivity against HEp-2 extracts in AD comparatively to SD (at 1:100 dilution, P = 0.031) whereas the latter presented higher IgG2 reactivity (at 1:100 dilution P = 0.0033).

Moreover, we observed that autoantibodies against human albumin (Fig. 5A), ds-DNA (Fig. 5 B) and human transferrin (Fig. 5 C) in dogs' sera were dominated in all analyzed groups by the IgG2 isotype. The reactivity of IgG2 autoantibodies was stronger in SD relatively to HND (P < 0.0001) and to AD (P < 0.003). The reactivity of the IgG1 isotype with albumin and ds-DNA, even though statistically non-significant, was recorded at a higher frequency in AD and SD groups comparatively to HND. A significant increase of IgG1 and IgG2 anti-transferrin antibodies is recorded in SD as well as in AD in comparison with healthy dogs from endemic areas. The relative

reactivity of IgG2, but not of IgG1, anti-transferrin was significantly higher in SD comparatively to AD ($P = 0.0004$).

In order to better characterize the specificity of IgG1 and IgG2 autoantibodies, we checked by competitive ELISA whether their binding to leishmanial antigens in *L. infantum*-ELISA experiments was specific. Two-fold serially diluted serum samples obtained from AD and SD were pre-incubated with a fixed amount of leishmanial antigens then added to HEp-2-coated plates. Serum dilutions without inhibitor were used as controls. Results show that, while the degrees of inhibition of IgG2 binding by the leishmanial antigens were similar between groups (at dilution 1:150; 49% of inhibition in AD and 48% in SD), those of the IgG1 were more important in SD (70% and 30% for SD and AD, respectively), suggesting that the proportion of cross-reactive IgG1/non cross-reactive autoantibodies in more important in SD than in AD (Fig. 6 A). The addition of higher amounts of leishmanial antigens did not result in complete (100%) inhibition of antibody binding to HEp-2 coated plates. Moreover, the lower percentage of IgG2 inhibition comparatively to IgG1 (48% *versus* 70%) in SD (Fig. 6 B) is in favor that the relative proportion of cross-reactive IgG1 autoantibodies is higher than that of their IgG2 counterpart. In the opposite, the proportion of cross-reactive IgG2 autoantibodies in AD is more important than its counterpart IgG1.

3.3. Western blot analysis

The reactivity of AD and SD serum samples towards HEp-2 antigens was also compared by WB to check whether dog's autoantibodies have targeted or not the same proteins. Examples of IgG1 (lanes a) and IgG2 (lanes b) reactivity, illustrated in Fig. 7, showed that both groups produced highly heterogeneous immunodetection patterns. However, it is noteworthy that IgG1 antibodies from six out of ten AD seem to be more reactive than IgG2 antibodies (examples AD1, AD4 and AD7) whereas the opposite was observed for SD serum samples. IgG1 and IgG2 autoantibodies from late stage SD (high TCS, SD9 to SD12) seem also more reactive with HEp-2 proteins. IgG1 and IgG2 reactivity was mostly targeting the proteins with 72, 62, 46, 43, 35, 30, 23, and 16 kDa of

MW. The protein band with 16 kDa was more frequently reactive in AD group. Henriksson et al., (1998) analyzed by HeLa cell nuclear extract immunoblots the serum reactivity of ANA positive dogs and showed that a 43 kDa nuclear protein, previously described as hnRNP G, was the most frequently recognized autoantigen.

4. Discussion

We aimed the present study to analyze the specific and autoimmune repertoires of *L. infantum*-infected dogs for a better understanding of the parasite-induced development of the specific and non specific humoral immune responses. Indeed, many investigations of the specific antibody response have led to inconsistent results where (i) authors claimed that either IgG1 or IgG2 is the isotype marker of the asymptomatic infection status whereas (ii) others suggested that the specific antibody responses cannot be used as surrogate markers of the T cell immune responses or as an indication of the clinical status of infected dogs (Quinnel et al., 2003; Strauss-Ayali et al., 2007), highlighting the difficulties to establish a typical immunological response profile of *L. infantum*-infected dogs.

The results presented herein, which analyzes for the first time the IgG1-IgG2 humoral responses of *L. infantum*-infected domestic dogs living in Tunisian endemic areas, oppose the studies reporting higher levels of specific IgG1 or IgG2 during the subclinical phase of dog's infection but also those describing that AD and SD produce similar levels of specific IgG2 (de Freitas et al., 2012; Lima et al., 2017). Results obtained by ELISA based on native soluble *L. infantum* antigens showed that the levels of specific Tot IgG, IgG1 and IgG2 were constantly higher during the active clinical infection. The use of leishmanial LACK and LeIF recombinant proteins corroborated the obtained results despite a lower assay's sensitivity (except for LeIF-IgG2). An overall strong degree of correlation was observed between Tot IgG and IgG2 ODs but not with IgG1 ODs, in SD and AD groups. Moreover, we did not observe, as described by Quinnell et al. (2003), that the titers of specific IgG1 exceed those of IgG2 neither in SD nor in AD. Individual

specific IgG1 levels were constantly lower than those of IgG2 in all dogs (Almeida et al., 2005), except in one AD, two SD and interestingly in seven HND. The presence in HND of an exclusive IgG1 (31%, median OD = 0.4) or IgG2 (6.6%, median OD = 0.8) reactivity with leishmanial antigens, raises the possibility that, despite Tot IgG ODs lower than cut-off value, dogs may already have been infected with *L. infantum* and that blood sampling occurred at a time period where their immune status precedes that of AD. If our hypothesis was true, it would suggest that healthy dogs with undetectable Tot IgG but with positive IgG1 and/or IgG2 ODs, may be included in the AD group.

Several hypotheses were previously proposed to explain such inconsistencies that raised despite the use of the same *Leishmania* strain, the investigation of the same endemic areas and the use of very similar experimental ELISA protocols based on commercial polyclonal or monoclonal anti-dog antibodies produced from the same companies. Hypotheses were more often related to the use of polyclonal instead of monoclonal anti-IgG isotypes, considered less sensitive, rather than to differences in the degree of specificity of the *Leishmania*-antigenic preparations caused by the cross-reactivity with other environmental pathogenic antigens. In addition, data variations resulting from (i) technical differences among which the choice of the reference population and of the cut-off value (Greiner et al., 2000; de Arruda et al., 2013), and from (ii) biological differences such as blood sampling period which could lead to differences in dog's immune status, were also discussed in the literature.

It is noteworthy that the molecular mimicry between *Leishmania* and host antigens suspected in the development of autoimmunity, could also be responsible for the "non-specific binding" of human's or dogs' sera, affecting hence the specificity of the serology assays. Indeed, Argov et al. (1989) detected the presence of IgG autoantibodies against Sm, RNP and SS-B nuclear antigens in the sera of 83, 86, and 73% of hVL cases, respectively but in less than 25% of cutaneous leishmaniasis patients. Authors showed by competitive ELISA that the binding of human VL serum antibodies to RNP could be inhibited after prior incubation of serum with leishmanial membrane

antigens, implying that, besides the polyclonal activation of B lymphocytes, the production of autoimmune antibodies might have resulted from antigen cross-reactivity between leishmanial antigens and RNP. In addition, Louzir et al. (1994) reported an increase of polyreactive anti-tubulin, anti-actin and anti-myosin IgG natural autoantibodies in 86, 68 and 57% of Tunisian children with VL, respectively.

The production of circulating immune complexes (Lopez et al., 1996) and autoantibodies such as antinuclear antibodies (anti-DNA, RNA, nuclear proteins and their molecular complexes), and anti-mammalian basal membrane glycoproteins and cerebrosides, and anti-Histone, was previously described in *L. infantum*-infected dog's sera (Pateraki et al., 1983; Lucena and Ginel, 1998; Bell et al., 1997; Smith et al., 2004). Ferrer (1992) and Lucena et al. (1996) reported a 30 and 15.9% incidence of antinuclear antibodies in dogs with natural *Leishmania* infection, respectively. Pateraki et al. (1983) demonstrated by ELISA that 250 (95%) and 248 (94%) dogs out of 260 with cVL, all positive for *L. donovani* antigens, have anti-actin and anti-tubulin IgG autoantibodies, respectively. Ginel et al. (2008) reported that 88% of infected dogs with glomerulonephritis had high anti-histone antibodies. However, to our knowledge, no study analyzed the profile of IgG1 or IgG2 autoantibodies during the subclinical or the acute phase of dog's infection with *L. infantum.* Our results from ELISA-HEp-2 indicate the presence of an autoantibody activity in all groups including HND. Indeed, for the latter, we observed as described by Argov et al. (1989), investigating human VL, a relatively higher reactivity of endemic controls comparatively to the negative control group. This suggests that, if in case these dogs were previously infected, the polyclonal activation of B cells producers of natural antibodies occurred during the early stages of dog's infection.

Moreover, we observed that the reactivity of Tot IgG and IgG2 with nuclear and soluble HEp-2 extracts was significantly higher in SD, in terms of ODs and positive reactivity (%). Thus, 100% and 74% of tested SD presented Tot IgG and IgG2 serum binding to HEp-2 whereas only 43% and 68% of AD were positive. Conversely, seroprevalence of IgG1 subclass was higher in AD

(50%) than in SD (26%). Positive binding to HEp-2 proteins for SD group was independent of parasitological tests' results and was present in positive and negative serum samples. It was also present in AD serum samples reactive with *L. infantum* extracts, LACK and LeIF proteins, implying that the increase of autoantibody levels in these dogs was associated to *Leishmania* infection. In addition, we show that for the IgG1 isotype, the autoantibody response in AD was mainly directed against antigenic determinants that do not cross-react with *Leishmania* parasite (only 30% of inhibition by leishmanial antigens) whereas it was dominated by cross-reactive IgG1 antibodies in sick dogs (70% of inhibition by leishmanial antigens). For the IgG2 isotype, and for both dogs' groups cross-reactive and non cross-reactive autoantibodies seem to be similarly represented.

Furthermore, it has been previously reported that the development of autoimmune reactions in human and canine VL could contribute to multiple alterations among which normocytic anemia, renal, hepatic and vascular alterations. Thus, the increase of the concentration of circulating immune complexes and their deposition in kidney tissue could be responsible for necrosis and impairment of the renal function, one of the leading causes of death in cVL (Brandonisio et al., 1990; Ciaramella, P. and Corona, M., 2003; Baneth et al., 2008; Koutinas and Koutinas, 2014). Transferrin is a plasma protein that transports iron through the blood to the liver, spleen and bone marrow and low serum transferrin levels was previously associated with anemia and chronic liver disease. Toet et al. (2014) found that *Sarcoptes scabiei* infested pigs produce antibodies to albumin and to iron-binding proteins including ferritin and transferrin. The development of natural IgG autoantibodies against transferrin in *Leishmania*-infected dogs, particularly in SD, could be a possible mechanism that lowers protein and iron levels, decreases hemoglobin production (due to lack of iron), leading to anemia and impairment of liver function. Further investigations, such as the measure of iron, hemoglobin and transferrin concentrations in dogs' infected sera, are however required to check whether these autoantibodies may trigger the development of anemia or liver disease, characteristic features of cVL.

5. Conclusion

In this paper, ELISA results based on *L. infantum* native and recombinant proteins indicate that in a cohort of domestic dogs living in Tunisian endemic areas, the antibody titers of specific Tot IgG, IgG1 and IgG2 were constantly more elevated during the active phase of infection and that IgG2 is the predominant subclass present in infected AD and SD. The increase of autoantibodies' production starts since the subclinical stages and is characterized by relatively low levels of cross-reactive IgG1 antibodies. Conversely, IgG1 cross-reactive antibodies dominate the autoantibody repertoire of SD. Taken together, our findings are in favor that the discordance described in previous studies could result from low specificity caused by the interference of a variety of autoantibodies, including natural and cross-reactive antibodies.

Author Contributions

AS conceived the paper, designed and performed the ELISA and western blotting experiments, analyzed the data and wrote the paper. AC participated in drafting and writing the manuscript. RBE veterinary physician collected healthy dogs' blood samples and critically revised the paper. MM veterinary physician established clinical dog's diagnosis, collected blood samples and revised the paper. MG participated in manuscript's revision. All authors approved the final manuscript version.

Declaration of interest: none

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Figure legends

Figure 1. ELISA results of dog's serum samples reactivity with leishmanial antigens. (A) Comparison of the IgG, IgG1 and IgG2 level's between HND (n=45), AD (n=26) and SD (n=34). Each dot represents one individual dog's serum tested at dilutions 1:100 and 1:500. Horizontal lines represent the median value of each plot. The cut-off values for dilution 1:100 (dashed lines) were determined from 26 CTD (mean + 3 SD) and used to check for positive serum reactivity. The seroprevalence (%) of positive dogs is indicated for the dilution 1:100. The unpaired *t*-test of Student's was used to calculate significance levels between the OD values; *P* values from comparison between AD and SD groups are shown. Significant differences between non infected (HND) and infected dogs (AD or SD) were obtained for all antibody levels ($P < 0.0001$). (B) Correlations between the OD values of Tot IgG and IgG2 in SD (dilution 1:2500) and AD (dilution 1:500) groups. The Spearman's coefficient of correlation (r) and the *P* values are indicated on the graph. (C) Symptomatic *L. infantum*-infected dogs produce higher levels of specific IgG1 and IgG2 antibodies than AD against leishmanial antigens. Sera collected from 12 AD, 16 SD and 5 CTD were diluted at 1:100, 1:400, 1:1600 and 1:6400 and tested by ELISA for their reactivity towards leishmanial antigens. Results are expressed as mean of OD ± 1 standard error of the mean (SEM).

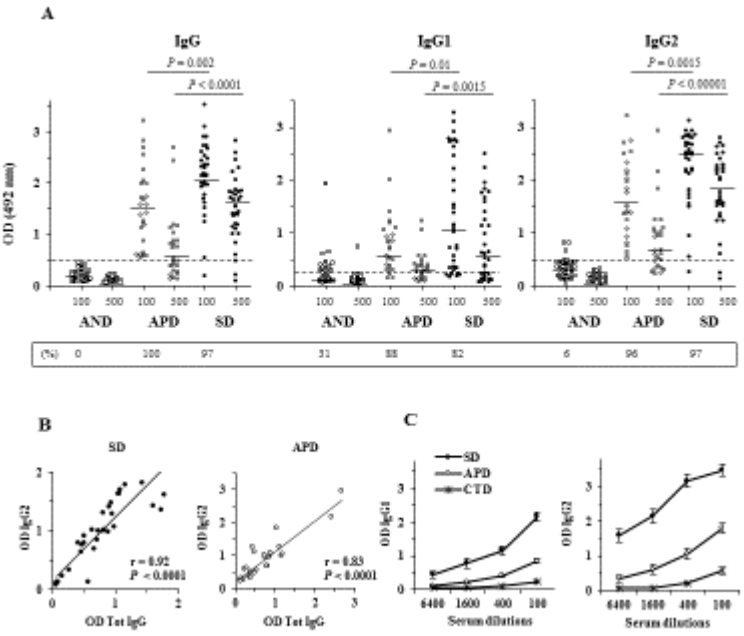

Figure 2. Reactivity of IgG1 and IgG2 antibodies against LACK and LeIF recombinant proteins. Sera from 12 HND, 20 AD and 15 SD were diluted at 1:100 and incubated on LACK (A, B) or LeIF-coated plates (C, D). Values in box plots reflect the median and the interquartile range of IgG1 (A, C) and IgG2 (B, D) serum reactivity. The boxes represent 95% of the values. Cut-off values were determined from 6 CTD dogs (mean + 2 SD). *P* values from comparison by the unpaired Student's t-test between AD and SD are shown. All comparisons between HND and AD or SD serum sample's reactivity were statistically significant ($P < 0.04$ with AD, and $P < 0.001$ with SD).

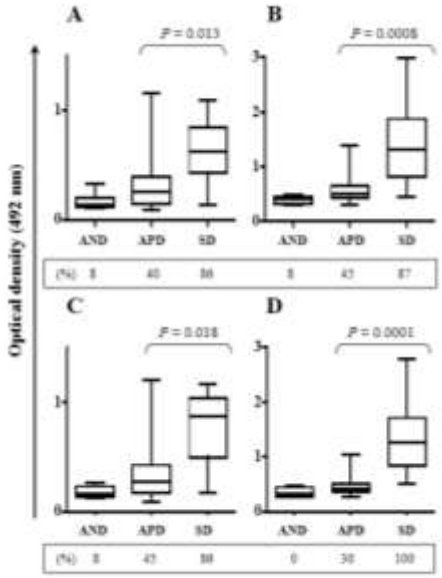

Figure 3. ELISA of IgG, IgG1 and IgG2 reactivity of dog's sera to HEp-2 cell extracts. In panel A, the reactivity of IgG, IgG1 and IgG2 antibodies was compared between HND (n=27), AD (n=22) and SD (n=34). Horizontal lines represent the plots' median values. The cut-off values (dashed lines for dilution 1:100) were determined from 22 CTD (mean + 3 SD) and used to check for serum positive reactivity. The seroprevalence (%) is indicated for the dilution 1:100. The unpaired *t*-test of Student's was used to calculate significance levels between OD values; *P* values from comparison between HND, AD and SD groups are shown. Panel B shows the correlations between the OD values of Tot IgG and IgG2 in SD and AD groups. Panel C shows the correlations between IgG2 reactivity with HEp-2 and *L. infantum* antigens. The Pearson's r coefficients and the *P* values are indicated on the graph.

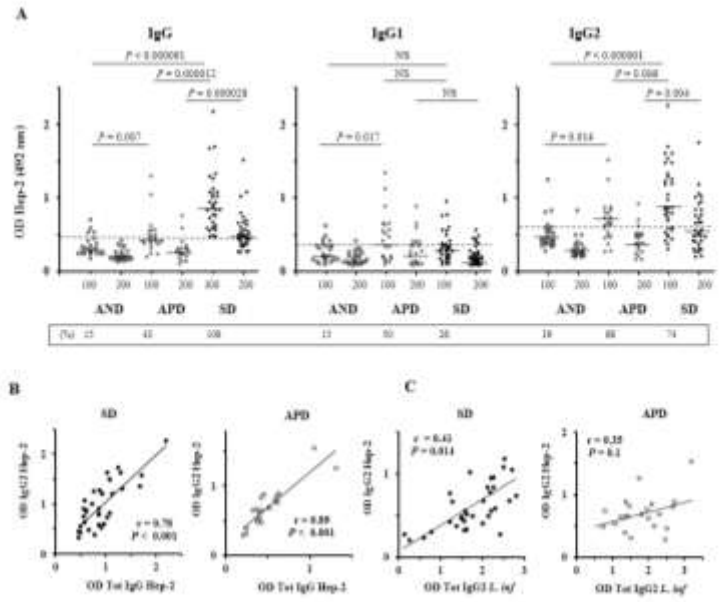

Figure 4. Asymptomatic *L. infantum*-infected dogs show higher reactivity of IgG1 antibodies against HEp-2 extracts than symptomatic dogs ($P < 0.04$) (A) whereas the latter present higher levels of IgG2 autoantibodies ($P < 0.003$) (B). Sera collected from 6 AD, 9 SD and 5 CTD were diluted at 1:100, 1:200, 1:400 and 1:800 and tested by ELISA. Results are expressed as mean of OD ± 1 SEM.

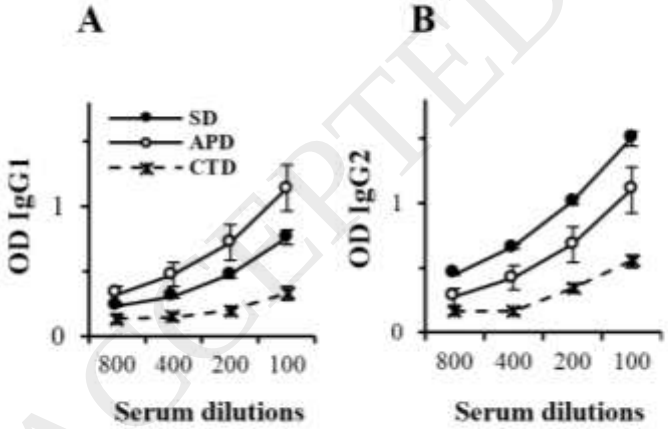

Figure 5. IgG1 and IgG2 reactivity to albumin (A), ds-DNA (B) and transferrin (C). Dogs' sera were from HND (n = 5 to 16), AD (n = 10 to 21), and SD (n = 10 to 32). Serum samples from CTD (n = 5 to 11) were used to establish cut-off values for positivity at a dilution of 1:100 (dashed lines).

Values given are the median OD_{492} of each group of dogs (horizontal lines). The Mann-Whitney U test was used for comparisons between groups. NS indicates non significant difference between groups. NS*: Comparison between only positive dogs was significant ($P < 0.05$).

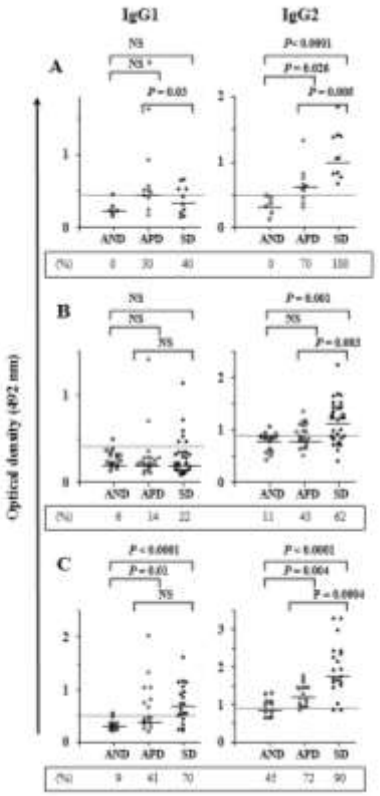

Figure 6. Competitive ELISA with HEp-2 coated plates. Dog's serum samples obtained from 8 AD (A) and 7 SD (B) which displayed anti-HEp-2 activity were incubated with (W) or without (WO) 100 µg/ml of leishmanial antigens. Sera were tested at two-fold serial dilutions from 1:150 to 1:1200. Results are expressed as mean ± 1 SEM of OD. The percentage of inhibition of IgG1 and IgG2 binding to HEp-2 coated wells by leishmanial antigens is provided for 1:150 serum dilution. Decrease in antibody binding to HEp-2 antigens was significant by paired Student's *t*-test for both groups and isotypes. *P* values are indicated in the figure for dilution 1:500.

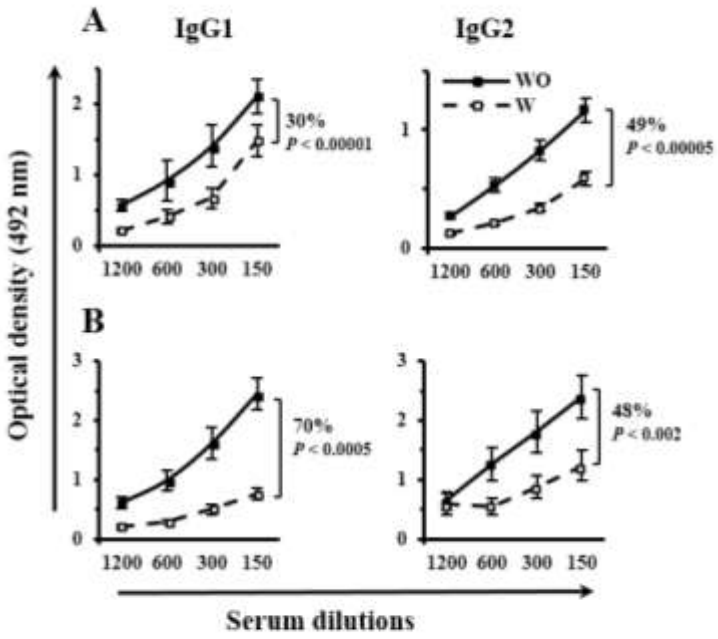

Figure 7. Immunodetection profiles of IgG1 (lanes a) and IgG2 (lanes b) antibody reactivity with HEp-2 proteins. Serum samples from 12 SD (panel A), 11 AD and 1 CTD (panel B) were diluted at 1:500 and tested against HEp-2 proteins after separation by electrophoresis on 15% SDS-acrylamide gels and blotting to nitrocellulose membranes. The TCS of SD samples is shown. Numbers indicate proteins' MWs. Serum samples from AD (AD1, AD4, AD5, AD7, and AD10) and from SD (SD7 and SD11) exhibited higher IgG1 reactivity than IgG2.