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Research paper

Circulating immune complexes levels correlate with the progression of canine leishmaniosis in naturally infected dogs

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ABSTRACT

Dogs are the main domestic reservoir of *Leishmania infantum*, and in cases of uncontrolled infection, a strong humoral immune response is elicited, which is inefficient against the parasites. Previous studies have suggested that an adequate antigen/antibody ratio, with a moderate prevalence of antigens with respect to the antibodies, could result in the formation of circulating immune complexes (CIC) in canine leishmaniosis (CanL). Deposition of these complexes in tissues has been associated with vasculitis, uveitis, arthritis, dermatitis and especially glomerulonephritis and renal failure. However, little is known about the relationship between the presence of CIC and disease progression. The aim of this study was to evaluate serum CIC level and its correlation with disease severity in infected dogs with different stages of disease and non-infected animals as a control. A total of 60 dogs were included in the study, classified according to the proposed LeishVet classification criteria: healthy non-infected (n = 13); healthy infected (n = 12); sick stage I (n = 9); sick stage II (n = 17); sick stage III (n = 8); and sick stage IV (n = 1). CIC were isolated from serum samples using a modified polyethylene glycol precipitation method, and their levels measured by ELISA and bicinchoninic acid protein assay. A nanoparticle tracking analysis was performed to investigate the relationship between the molecular size distribution of the CIC and disease progression. In conclusion, the results confirmed a positive association between CIC levels, their molecular size and disease progression that suggests a potential use of CIC as biomarkers of CanL.

1. Introduction

Canine leishmaniosis (CanL) is a global zoonotic disease caused by the parasite *Leishmania infantum* and transmitted by phlebotomine sand flies; the main arthropod vector is *Phlebotomus perniciosus* (Willen et al., 2018). CanL is endemic in the Mediterranean Basin, Central and South America and some regions of Asia. It is recognized as a major problem for both veterinary medicine and public health, given that dogs are the main domestic reservoir of *L. infantum* (Dantas-Torres, 2007; Galvez et al., 2010).

Macrophages infected with *Leishmania* parasites are ruptured due to hypertrophic growth leading to the release of the amastigote forms. These amastigotes trigger the humoral immune response, and in animals unable to limit the infection, this leads to a large and uncontrolled antibody production which is inefficient against parasites (Jamal et al., 2017). Persistent infection associated with prolonged presence of parasite antigens can result in the formation of circulating immune complexes (CIC) (Nydegger, 2007), formed mainly by IgG, IgM and complement system components in addition to antigens from the parasite (Poli et al., 1991). Defective clearance of these CIC by scavenging macrophages leads to their deposition in specific organs.

Several studies in animal models and also in humans have attempted to elucidate the role of CIC in the pathology of visceral leishmaniosis (VL) (Requena et al., 2000; Miles et al., 2005; Elshafie et al., 2007), suggesting that disease complications may be partly accounted by CIC deposition-related pathology, particularly nephritis (Poli et al., 1991; Lopez et al., 1996; Costa et al., 2003; Plevraki et al., 2006). CIC-related renal pathology plays a pivotal role in prognosis, and it has been

Abbreviations: BCA, bicinchoninic acid; CanL, canine leishmaniosis; CIC, circulating immune complexes; ELISA, enzyme linked immunosorbent assay; H, healthy; HI, healthy infected; IFAT, indirect fluorescent antibody test; NTA, nanoparticle tracking analysis; PEG, polyethylene glycol; SLA, soluble *Leishmania* antigens; SS, sick stage

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adopted as a major criterion for clinical staging of the disease in dogs (Paltrinieri et al., 2010; Solano-Gallego et al., 2011; Roura et al., 2013). Immune complexes have been detected in the sera of *Leishmania*-infected dogs, and the pathogenesis of renal lesions has been mainly attributed to CIC deposition and subsequent glomerular injury (Tafuri et al., 1989; Poli et al., 1991; Nieto et al., 1992; Margarito et al., 1998; Solano-Gallego et al., 2003).

Several assay systems have been developed for detection of soluble complexes (Sobel et al., 1975; Hay et al., 1976; Digeon et al., 1977; Levinsky and Soothill, 1977), with different success rates. Previous studies have highlighted the importance of CIC in the pathogenesis of a variety of systemic disorders such as autoimmune diseases (Abrass et al., 1980; Nydegger and Davis, 1980; Bernstein et al., 1994), allergic diseases (Paganelli et al., 1981; Park and Nahm, 1998) and infectious diseases (Miles et al., 1993; Sengupta et al., 2002; Koraka et al., 2003). This suggests that CIC may be considered potential biomarkers of disease activity due to their diagnostic and prognostic value (Urbaniak-Kujda, 1996; Brunner and Sigal, 2001; Jaiswal et al., 2018; Thanadetsuntorn et al., 2018). In this sense, quantification of CIC in cases of CanL could be an alternative disease biomarker not only for diagnosis but also for tracking disease progression and potentially for monitoring the success of treatment in dogs.

Thus, the aim of this study was to determine the presence of CIC in naturally infected dogs suffering from clinical CanL at different stages and to investigate the correlation between CIC levels and disease progression.

2. Materials and methods

2.1. Experimental population

The experimental population consisted of 60 dogs brought by their owners to the hospital clinic (Veterinary Teaching Hospital, Universidad Complutense de Madrid, Spain) and breeder and hunting dogs at their respective kennels undergoing a CanL check-up over the period December 2013 to June 2014. The owners of the selected dogs were informed beforehand about the study protocol and gave their informed consent.

Animals were classified into the following leishmaniosis clinical stages according to the LeishVet classification (Solano-Gallego et al., 2009, 2011) based on clinical signs, clinical-pathologic abnormalities and serologic status: healthy non-infected (H, n = 13); healthy infected (HI, n = 12); sick stage I (SS1, n = 9); sick stage II (SS2, n = 17); sick stage III (SS3, n = 8); and sick stage IV (SS4, n = 1).

Selection criteria included male or female dogs, not previously vaccinated against *L. infantum* or treated in the previous two months with any leishmanicidal/leishmanistatic drugs. Dogs infected with other vector-borne pathogens (i.e. *Ehrlichia* spp., *Babesia* spp. or *Dirofilaria* spp.) were excluded to avoid misinterpretation of clinical signs and/or clinical-pathologic abnormalities.

2.2. Serum sample collection

Approximately 8 mL of whole blood from each dog were collected and kept at room temperature for approximately 30 min and then centrifuged at 1000 × g for 10 min at 4 °C. After centrifugation, a minimum volume of 4 mL of serum per animal was obtained and stored at −20 °C until use.

2.3. CIC isolation

Serum samples were processed for CIC isolation using a modification of a previously reported method (Raja et al., 1995). Briefly, samples were centrifuged and the upper layer collected and mixed with an equal volume of 7% polyethylene glycol (PEG; Sigma-Aldrich, St Louis, MO, USA) and kept overnight. Afterwards, PEG-precipitated CIC were pelleted by centrifugation and then reconstituted in 0.01 M phosphate buffered saline (PBS) and stored at −80 °C for further use. CIC protein content was estimated by using a bicinchoninic acid (BCA) protein assay kit (Thermo Fisher Scientific, Waltham, MA, USA).

2.4. L. infantum culture and soluble antigen preparation

L. infantum (MCAN/ES/1996/BCN/150/MON-1) parasites were grown at 26 °C in Schneider's medium (Sigma-Aldrich) supplemented with 20% heat-inactivated fetal bovine serum (Sigma-Aldrich), 20 mM L-glutamine, 200 U/mL penicillin and 100 µg/mL streptomycin at pH 7.4 (Fernandez-Cotrina et al., 2013).

Soluble *Leishmania* antigen (SLA) was prepared from stationary-phase promastigotes of *L. infantum*, as reported previously (Iborra et al., 2004). Briefly, 2×10^8 promastigotes were washed three times in cold sterile PBS and subjected to six cycles of freezing and thawing. After five cycles (30 s at 38 MHz) of ultrasonication (Labsonic® M, Sartorious, Goettingen, Germany), the suspension was centrifuged at 8000 xg for 30 min at 4 °C and the supernatant containing the SLA was collected. Protein concentration was estimated by the Bradford method (Bio-Rad, Hercules, CA USA), and 500 µL aliquots were stored at −80 °C.

2.5. Levels of CIC

An enzyme-linked immunosorbent assay (ELISA) procedure was used to measure PEG-precipitated CIC. Briefly, microtiter immunoassay plates (NUNC, Roskilde, Denmark) were coated with SLA (10 µg/mL in 0.01 M PBS) overnight at 4 °C. Microplates were washed four times with PBS containing 0.1% Tween 20 (PBS-T) and blocked with 200 µL/well of PBS-T supplemented with 1% BSA (Sigma-Aldrich) at room temperature (RT). After washing, microplates were incubated with a 1:100 dilution of precipitated CIC for 1 h at RT. Microplates were then washed and incubated with HRP-conjugated anti-dog IgG (Bethyl Laboratories, Montgomery, TX USA) (1:10,000) at RT for 1 h. After washing, microplates were developed with a solution of Sigma Fast o-phenylene diamine dihydrochloride (OPD) and H_2O_2 in phosphate-citrate buffer. After 30 min, the reaction was stopped by adding a solution of 2 N H_2SO_4, and microplates read at 450 nm in an ELISA microplate spectrophotometer (Bio-Rad).

All samples were tested in duplicate and the mean value was recorded. Mean OD + 2 Standard Deviation of the control group (group H) was considered as the cutoff value; any sample exhibiting absorbance above the cutoff value was considered positive.

2.6. Determination of leishmania-specific antibodies in serum samples by immunofluorescence antibody testing (IFAT)

Specific IgG antibodies against in-house cultured promastigotes were evaluated as reported elsewhere (Mancianti and Meciani, 1988), using serial dilutions from 1:50 to 1:12,800; seropositivity was defined by a cut-off ≥ 1:100.

2.7. Analysis of particle distribution

Absolute particle distribution (size and concentration) was analyzed by nanoparticle tracking analysis (NTA) using a NanoSight NS300 (Malvern Panalytical, Malvern, UK) equipped with a sample chamber and 405 nm laser.

PEG-precipitated CIC were injected into the sample chamber with sterile syringes until the liquid reached the tip of the nozzle. NTA measurement conditions were: temperature = 22 °C; viscosity = 0.95 cP; frames per second = 21; measurement time = 91 s; three recordings performed per sample.

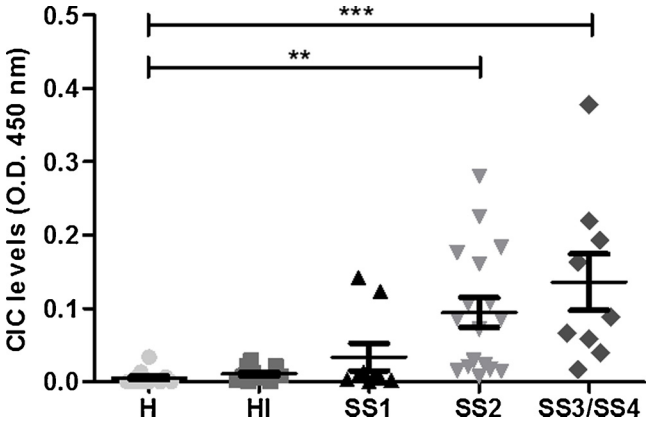

Fig. 1. Levels of polyethylene glycol (PEG)-precipitated circulating immune complexes (CIC).
CIC quantification was performed in PEG-precipitated serum samples from different groups (healthy non-infected (H); healthy infected (HI); sick stage I (SS1); sick stage II (SS2); sick stage III / sick stage IV (SS3/SS4)). Data show values of individual animals and mean ± SEM. Low levels of CIC were detected in groups H and HI while statistically significant differences, determined using nonparametric Student´s *t*-test (Mann-Whitney), were observed between groups SS2 and SS3/SS4 in comparison to healthy controls (**$p < 0.01$; ***$p < 0.001$).

2.8. Statistical analysis

Data from groups SS3 and SS4 were merged and analyzed together due to the relatively low number of animals classified in the group SS4 included in the study.

Nonparametric Student's *t*-tests (Mann-Whitney) were performed with GraphPad Prism 5.0 software (GraphPad Software, San Diego, CA, USA). Data were expressed as mean ± standard error mean (SEM), and differences were considered statistically significant when $p < 0.05$.

Correlation coefficient (r value) and statistical significance of pairwise comparisons between PEG-precipitated CIC levels and IFAT titer or total protein content were determined using Spearman's correlation.

3. Results

3.1. Measurement of PEG-precipitated CIC

PEG-precipitated CIC levels are shown in Fig. 1. No CIC were detected either in group H (healthy non-infected animals) or in group HI (healthy infected animals).

Statistically significant differences were observed between groups SS2 ($p < 0.01$) and SS3/SS4 ($p < 0.001$) compared to group H. No statistically significant differences ($p = 0.052$) were detected between group SS1 and the control group (H).

3.2. Protein content in PEG-precipitated CIC

PEG-precipitated CIC protein content is shown in Fig. 2A. Protein content was significantly higher ($p < 0.05$) in sick groups (SS1, SS2 and SS3/SS4) compared to the control group (H).

A significant positive correlation was obtained between protein concentration and levels of PEG-precipitated CIC ($r = 0.747$; $p < 0.001$) (Fig. 2B).

3.3. IFAT analysis

Leishmania-specific IgG determined by IFAT ranged from negative (no reactivity) to 1:6400. Statistically significant differences in IgG titers were observed between groups SS1 ($p < 0.05$), SS2 and SS3/SS4 ($p < 0.001$) compared to group H. Dogs in groups SS2 and SS3/SS4 showed the highest antibody titers (Fig. 3A).

Fig. 3B shows a statistically significant positive correlation between IFAT titers and levels of PEG-precipitated CIC ($r = 0.754$; $p < 0.001$).

3.4. Particle distribution

The analysis of absolute particle distribution (size and concentration) in PEG-precipitated serum samples is shown in Fig. 4.

Large amounts of small, non-aggregated particles (70 nm) were observed in groups H, HI and SS1. In contrast, groups SS2 and SS3/SS4 showed a size distribution ranging from 100 to 400 nm, associated with larger protein aggregates, mainly detected at 100, 190, 280, and 400 nm.

4. Discussion

The source of CIC as a consequence of *Leishmania* infection has been associated with formation of huge aggregates of parasite proteins, anti-*Leishmania* IgG and IgM and to a lesser extent complement components (Mancianti et al., 1989; Lucena et al., 1994). In this sense, their deposition in specific organs has been determined to be one of the main causes responsible for tissue damage, related to renal failure and eventually death in CanL (Costa et al., 2003; Ordeix et al., 2005). Although different published studies have suggested that the concentration of CIC is related to disease progression (Margarito et al., 1998; Miles et al., 2005), there have been no studies directly correlating clinical signs in CanL with CIC concentration.

To this purpose we developed a method to isolate CIC and quantify their levels in serum samples obtained from dogs naturally infected with *L. infantum*. Although the method exclusively provides levels of PEG-precipitated CIC based on a colorimetric assay and expressed in optical densities, it provided a useful tool for measuring the concentration in serum samples, enabling comparison between different groups. This study shows the importance of implementing a robust, fully validated method to quantify them.

This preliminary approach to measure the CIC levels enabled us to establish a statistically significant correlation between immune complexes and pathology stage in animals infected with *L. infantum*. As expected, healthy non-infected and healthy infected animals did not show CIC, while there was a direct relationship of CIC levels and disease progression in the sick dogs, in accordance with results demonstrated for other pathologies (Tuzun et al., 2004; Sheerin et al., 2006). The presence of CIC has been associated with complications in severely ill individuals suffering from different infectious diseases (Goedvolk et al., 2003; Monsalvo et al., 2011; Thomas et al., 2012).

IFAT is one of the most commonly used serologic methods to diagnose leishmaniosis, considered the "gold standard" test for *Leishmania* infection (Maia and Campino, 2008; Reis et al., 2009). Its correlation with clinical signs and the deposition of CIC has been investigated in-depth (Zatelli et al., 2003; Aresu et al., 2013; dos Santos et al., 2013; Rigo et al., 2013; Koutinas and Koutinas, 2014). In this sense, it is widely known that dogs with the highest level of antibodies tend to have the worst clinical manifestations, although some exceptions are not infrequent, as other authors have reported (Dantas-Torres et al., 2006; Miró et al., 2007). In our study, we saw a positive correlation between IFAT titers and CIC levels of animals in groups SS2 and SS3/SS4, as confirmed by statistical analyses ($p < 0.001$).

Another important issue in determining pathogenicity of CIC could be related to their molecular size, as suggested in previous studies in human immune complex diseases and animal models (Cochrane and Hawkins, 1968; Levinsky et al., 1977; Tung et al., 1981; Mageed et al., 1991; Korolevskaya et al., 2014). In addition to their conformation and charge, the deposition site of the immune complexes is related to their size, which has been shown to play a relevant role in the glomerular pattern of injury in *Leishmania* infected dogs (Marcussen et al., 1989; Poli et al., 1991; Nieto et al., 1992; Costa et al., 2003; Zatelli et al.,

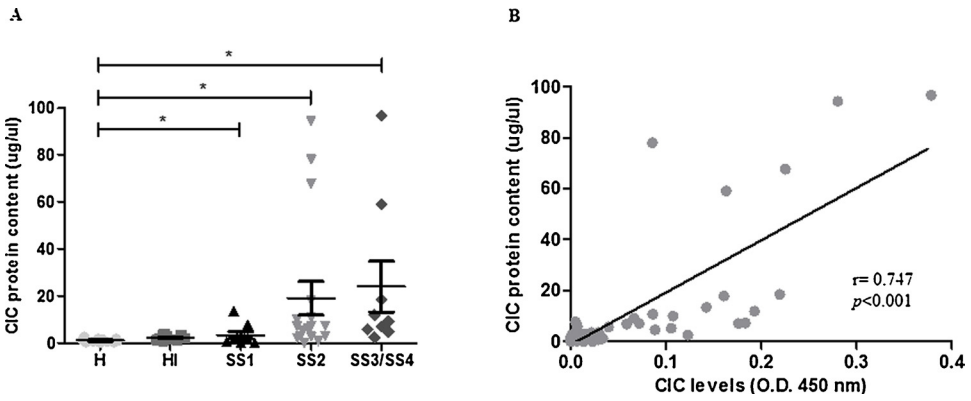

Fig. 2. Polyethylene glycol (PEG)-precipitated circulating immune complexes (CIC) protein content (A) and correlation with the levels of PEG-precipitated CIC (B).

A. CIC protein content in PEG-precipitated serum samples from different groups (healthy non-infected (H); healthy infected (HI); sick stage I (SS1); sick stage II (SS2); sick stage III / sick stage IV (SS3/SS4)), determined by BCA protein assay kit. Data show individual values and mean ± SEM. Statistical significance determined using nonparametric Student´s *t*-test (Mann-Whitney) in comparison to healthy controls for each group is indicated ($*p < 0.05$).

B. Correlation between protein concentration (μg/μl) and levels (O.D. 450 nm) of PEG-precipitated CIC. Spearman´s correlation ($r = 0.747$, $p < 0.001$).

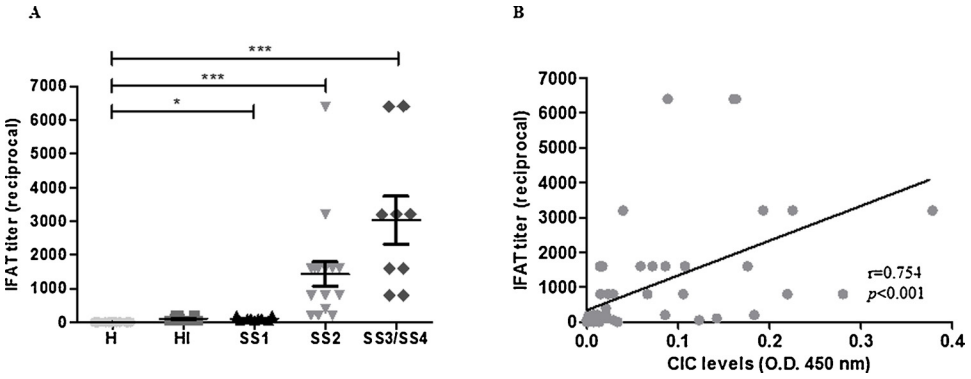

Fig. 3. Immunofluorescence Antibody Testing (IFAT) analysis (A) and correlation between IFAT and polyethylene glycol (PEG)-precipitated circulating immune complexes (CIC) levels (B).

A. Reciprocal IFAT titers/animal of different groups (healthy non-infected (H); healthy infected (HI); sick stage I (SS1); sick stage II (SS2); sick stage III / sick stage IV (SS3/SS4)) and mean ± SEM values. Comparison between groups was determined by nonparametric Student´s *t*-test (Mann-Whitney) in comparison to healthy controls for each group. Statistical significance is indicated ($*p < 0.05$; $***p < 0.001$).

B. Correlation between IFAT titer and levels (O.D. 450 nm) of PEG-precipitated CIC. Spearman´s correlation ($r = 0.754$; $p < 0.001$).

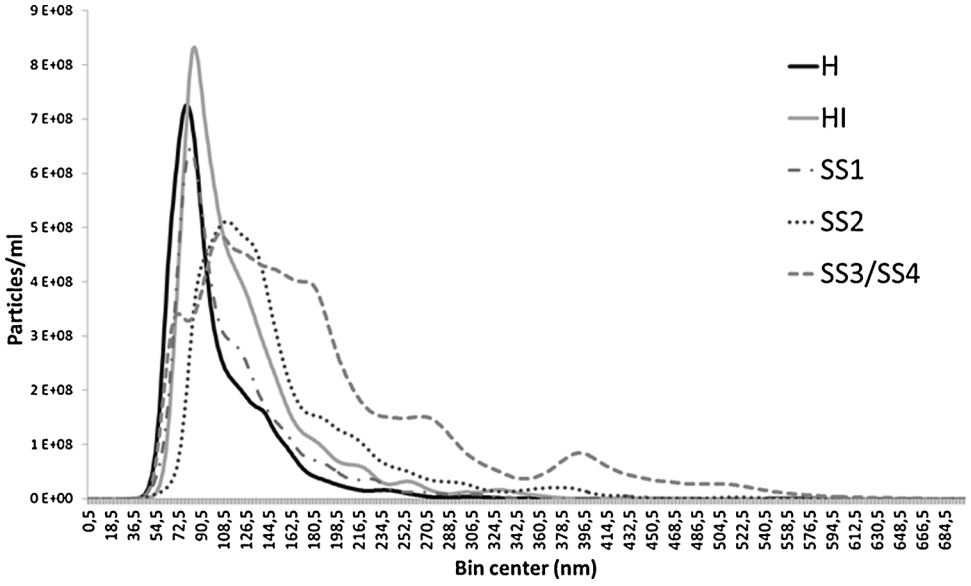

Fig. 4. Particle distribution (size and concentration) in polyethylene glycol-precipitated serum samples from the five groups of animals. The analysis of particle distribution was evaluated by Malvern Panalytical NanoSight NS300 technology. The chromatogram confirms that samples of groups: sick stage II (SS2) and sick stage III / sick stage IV (SS3/SS4), contain larger protein aggregates and an increase in the concentration of precipitated particles (high size, expressed in nm), in contrast with higher concentrations of small particles (low size, expressed in nm) observed in healthy non-infected (H); healthy infected (HI) and sick stage I (SS1) groups.

2003). As far as we know, there have been no previous studies reporting CIC size in CanL or correlating their size and disease severity. Thus, we applied NTA technology, a powerful technique that is particularly valuable for analyzing protein aggregates, to study CIC size in PEG-precipitated serum samples (Filipe et al., 2010). The data revealed that dogs with more severe clinical signs (groups SS2 and SS3/SS4) presented larger-sized protein aggregates, whereas a higher quantity of smaller-sized aggregates was observed in dogs included in groups H, HI and SS1. These results demonstrate a clear positive correlation between the clinical stage of the disease and the size of precipitated CIC providing additional evidence about their pathogenic role, as has been suggested in previous published studies (Mannik, 1980; Nangaku and

Couser, 2005; Korolevskaya et al., 2014).

Accurate assessment of CanL disease severity is necessary to support veterinarians, not only to confirm *Leishmania* infection but also to follow-up on the response to treatment. This would enable selection of appropriate therapeutic approaches and give a realistic prognosis to apparently healthy and sick dogs (Solano-Gallego et al., 2017; Maia and Campino, 2018). Despite the recent advances made in biomarkers related to *Leishmania* pathogenesis in different organs and tissues, due to invasive sampling and limited access to tools for evaluation of biological markers, most cannot be used in a laboratory setting (Maia and Campino, 2008). Nonetheless, and taking into account the above results, a non-invasive method to evaluate CIC levels (i.e. characterization of serum samples) would be a valuable tool to not only diagnose but also predict disease progression and potentially monitor the success of treatment in dogs. Furthermore, measurement of CIC as a biomarker for disease progression may provide interesting information regarding the ability of vaccines or immunotherapy treatments to control the disease. Undoubtedly, although other aspects about the origin and composition of CIC remain unknown, their relationship with disease progression has been definitively established.

5. Conclusion

This study reports a method to estimate CIC levels in dogs infected with *Leishmania* and demonstrates a direct correlation between those levels and the disease stage. Nevertheless, more studies are required to not only improve detection of CIC, but to further characterize them. To better understand the specific role of immune complexes in CanL and disease progression, it is crucial to determine the diagnostic, prognostic, and pathogenic importance of CIC. Thus, the future for the use of CIC as a biomarker for monitoring disease progression in CanL is promising.

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Declaration of competing interest

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